SHI/STY-Family Members Redundantly Regulate Auxin Homeostasis in Basal and Higher Plants

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Abstract

The hormone auxin plays fundamental roles in plant development by stimulating growth and differentiation of cells and tissues. Auxin action appears to rely on the formation of auxin gradients and/or concentration maxima, in large created by polar auxin transport. However, recent studies suggest that auxin synthesis also commence at sites of high auxin signaling and that spatiotemporal control of auxin biosynthesis may be required in the formation or maintenance of certain auxin maxima. The SHI/STY-family member STYLISH1 (STY1) has previously been shown to induce the activity of the auxin biosynthesis gene *YUCCA4* in lateral organs of *Arabidopsis thaliana*. This thesis describe the functional characterization of members of the embryophyte specific *SHI/STY*-family in the model species *A. thaliana* and *Physcomitrella patens*. Phenotypic analysis of *A. thaliana* plants carrying mutations in *SHI/STY*-family genes revealed that the gene products act highly redundant and in a dose-dependent manner in flower and leaf development. Protein studies showed that SHI/STY proteins regulate auxin biosynthesis by acting as transcriptional activators interacting with the *YUC4* promoter. Additionally, promoters of other auxin biosynthesis-related genes were activated by STY1. The sequence targeted by STY1 was identified by comparing target gene promoters. Mutations in the identified element abolished STY1-*YUC4* interaction in yeast. A conserved promoter element was also discovered in most *SHI/STY* members, including *STY1*. This element was shown to be essential for gene expression in most aerial organs and could be the target of AP2/ERF-family members. Identical expression patterns and knock-out mutant phenotypes of the moss *P. patens SHI/STY*-family members indicated that they act redundantly, as in *A. thaliana*. The knock-out and overexpressor lines showed phenotypes related to reduced and increased auxin levels, respectively, and the overexpressor lines indeed displayed elevated auxin levels. The *SHI/STY* genes of moss and higher plants are very similar and we suggest that they perform conserved functions.

Keywords: Arabidopsis thaliana, auxin, biosynthesis, *Physcomitrella patens*, plant development, redundancy, *SHI/STY*-family, *STYLISH1*, transcription factor, transcriptional regulation

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Kuusk, S., Sohlberg, J.J., Eklund, D.M. & Sundberg, E. (2006). Functionally redundant *SHI* family genes regulate *Arabidopsis* gynoecium development in a dose-dependent manner. *The Plant Journal* 47, 99-111.

II Eklund, D.M., Ståldal, V., Cierlik, I., Eriksson, C., Hiratsu, K., Ohme-Takagi, M., Sundström, F. J., Ezcurra, I. & Sundberg, E. The *Arabidopsis thaliana* STYLISH1 protein acts as a transcriptional activator regulating auxin biosynthesis. (manuscript).

III Eklund, D.M., Cierlik, I., Ståldal, V., Chandler, J & Sundberg, E. Expression of *SHORT INTERNODES/STYLISH* family genes in auxin biosynthesis zones of aerial organs is dependent of a GCC-box-like regulatory element. (manuscript).

IV [§]Eklund, D.M., [§]Thelander, M., Landberg, K., Ståldal, V., Nilsson, A., Johansson, M., Valsecchi, I., Kowalczyk, M., Ronne, H. & Sundberg, E. *Physcomitrella patens* orthologues of the *Arabidopsis SHI/STY/LRP1* genes induce auxin accumulation and affect growth and development in moss. (manuscript).

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§ Indicates shared first authorship

The contribution of Magnus Eklund to the papers included in this thesis was as follows:

I Lab-work such as Y2H, cloning and sequencing of *SRS8* and *PpSHI1*.

II Participated in planning of the project. Made the majority of lab-work except most qRT-PCRs. Made the majority of data analysis. Wrote the 1st draft.

III Came up with the idea. Made the majority of lab-work and bioinformatics. Made the majority of data analysis. Wrote the 1st draft.

IV Participated in planning of the project. Made lab-work including cloning the genes, planning and building of all constructs, some tranformations, RT-PCR and PCR verifications. Participated in analyzing data. Participated in writing the manuscript.

1 Introduction

In most plants, the fertilized egg cell, the zygote, undergoes a programmed pattern of cell divisions to produce the mature embryo, a rudimentary plant body consisting only of an embryonic axis and two cotyledons (if it is a dicot; reviewed in Willemsen and Scheres, 2004). This structure then develops into a seedling and then to an adult plant defined by, in some cases, thousands of organs and structures, e.g. roots, leaves and flowers. As these organs are not present in the embryo, or in the seedling, plants must initiate formation of most of its organs during postembryonic development and adult life. This is in sharp contrast to development of the body of most animals, where organs are initiated during embryogenesis and where postembryonic development mostly include enlargement and development of already existing organs. These differences of developmental strategy underline the fundamental differences in life style. Plants being sessile, permanently attached to their growth substrates, and are thus forced to deal with environmental factors, e.g. light and temperature fluctuations, water and nutrient supply, predators and mechanic stress, by adapting and shaping their body plan.

To understand how a zygote can give rise to a complex multicellular organism and by which mechanisms this organism constantly grow and develop new tissues and structures from preexisting structures, in response to genetic and environmental factors, is a goal for plant developmental biologists. Developmental biology thus aims to find, describe and understand the cellular, biochemical and molecular mechanisms making an organism grow and develop.

One requirement for multicellular growth is the communication between cells, tissues and organs, a communication that in part depend on long-range transport of signaling compounds, usually referred to as hormones, and also on compounds known as morphogens, forming concentration gradients that

mediate a developmental fate (reviewed in Bhalerao and Bennett, 2003). In general, plant cell fate is not determined by clonal lineage as in other organism groups, but rather by position, suggesting that adjacent cells, as well as more distantly located cells, provide the information necessary for determining cell fate.

This thesis describes the functional characterization of several members of the *STYLISH/SHORT INTERNODES* (*SHI/STY*) gene family in the angiosperm model *Arabidopsis thaliana* and the bryophyte model *Physcomitrella patens*. It deals with the mechanisms underlying their redundant influence on fundamental features of plant development, such as synthesis of the developmentally instructive plant hormone auxin. Finally, it also discusses the ancestral role of auxin by comparing the impact of *SHI/STY* function in angiosperm and bryophyte development.

1.1 Hormonal regulation of plant development

Plant hormones, also referred to as phytohormones or plant growth regulators, are small molecules of diverse origin that affect plant physiology at concentrations as low as the nano/picomolar range. The concept of hormones in plants originates in the characterization of hormones in animals models. In animals, hormones are molecules that signal between different regions of the body, hence, the signaling compound is created and released by a cell in one region and then transported, often in the blood stream, to another cell where the signal is perceived. However, in addition to long range transport-mediated signaling, plant hormones can also be produced at a specific site to increase local signaling in that specific tissue (Davies, 1995).

Plants use a wide array of different hormones, e.g. auxins, cytokinins (CKs), brassinosteroids (BRs), abscissic acid (ABA), ethylene (ET) and gibberellins (GAs), to send signals during virtually all physiological situations. The above mentioned classes of hormones have been shown to regulate major aspects of growth and development in *A. thaliana,* but in addition to these there are, however, many other hormones like those functioning mainly in pathogen responses and defense signaling, e.g. salicylic acid (SA) and jasmonic acid (JA).

1.1.1 Auxin

Auxins were first discovered when the bending of plants towards light was studied (Darwin and Darwin, 1880). The authors noted that the perceived information of light in one area of a grass coleoptile (the sheet covering the emerging shoot) is transported and causes changes of growth in another area,

resulting in bending. The messenger was later separated from plant tissues and it was shown that it retained its growth promoting activity (Wendt, 1926). Three different auxins were subsequently identified in plants and the structures were resolved, although only one of these structures, that of Indole-3-acetic acid (IAA), was confirmed to be correct (reviewed in Teale et al., 2006).

Auxin is a generic name for a group of compounds found in plants, humans and in microorganisms and of which IAA appear to be the most important in plants. Hence, also other endogenous compounds, e.g. 4-chloro-indole-3-acetic acid (4-Cl-IAA), phenylacetic acid (PAA) and indole-3-butyric acid (IBA) can act as active auxins in plants (Woodward and Bartel, 2005). There are also several synthetic compounds, e.g. 4-amino-3,5,6-trichloropicolinic acid (picloram), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 1-naphtaleneacetic acid (1-NAA), acting as active auxins when applied to plants (Woodward and Bartel, 2005).

Forward and reverse genetic studies have identified and characterized several molecular components regulating auxin synthesis, transport, perception and signaling. However, no mutant with total auxin deficiency has been identified and hence active auxin appears to be essential for plant growth and development (reviewed in Teale et al., 2006; Benjamins and Scheres, 2008). Moreover, auxin shows characteristics of a traditional hormone, as defined in animals, because it is transported from its source to a site of action where it binds a receptor. But it also resembles a classical morphogen, like the ones found in animals, because it regulates development in a dose-dependent manner through concentration gradients and concentration maxima (reviewed in Bhalerao and Bennet, 2003). Even so, the term hormone is generally accepted and will be used throughout this thesis.

Auxin has been connected to virtually all aspects of plant development. A few of these auxin-influenced processes are establishment of apical-basal polarity during embryogenesis, various tropisms (growth responses to environmental factors), lateral organ initiation, patterning of the vasculature, cotyledon formation, leaf morphogenesis, root and shoot architecture, lateral root formation, gynoecium/flower development, apical dominance and regulation of phyllotaxy (reviewed in Laux et al, 2004; Chandler, 2008, 2009; Benjamins and Scheres, 2008; Casimiro et al., 2003; Balanzá et al., 2006; Flemming, 2005). In the above-mentioned processes differential distribution of auxin and changes in local auxin levels acts as instructive signals mediating cellular differentiation and growth.

Important for practical work with auxin is to understand that exogenous application of a hormone and the subsequent evaluation of its effects can be misleading because it might be applied in a non-physiological concentration or at a site where it normally should not be. Auxin in high concentrations has been shown to induce ethylene production which e.g. can reduce growth and induce abscission (e.g. abortion of leaves) and can eventually cause death. Too much exogenous auxin will thus have negative effects on growth, while presumed physiological concentrations will enhance growth (Bandurski et al., 1995). Too high endogenous IAA levels will also have these affects and thus the cellular levels of free IAA need to be strictly regulated (Bandurski et al., 1995).

Auxin cross-talk to other hormones

Auxin signaling activate the expression of genes rate-limiting for ethylene biosynthesis (Abel et al., 1995; Tsuchisaka and Theologis, 2004), and ethylene stimulate auxin biosynthesis and transport e.g. to create an auxin signaling maximum in the root elongation zone (Romano et al., 1993; Stepanova et al., 2005, 2007, 2008; Ruzika et al., 2007; Swarup et al., 2007). Auxin-ethylene cross-talk is important for many aspects of development and has been extensively investigated in lateral root (LR) formation and development (Swarup et al., 2002; Casimiro et al., 2003; Negi et al., 2008; Ivanchenko et al., 2008).

The pleiotropic signaling defects of many auxin-response mutants suggest extensive cross-talk between auxin and signaling pathways of other hormones (reviewed in Swarup et al., 2002). The auxin-ethylene interactions in root growth are therefore just one of many examples of complex cross-talk in hormone signaling. E.g. BR can also interact with auxin in LR development (Bao et al., 2004) and, interestingly, auxin often act synergistically with BR signaling and in some cases auxin response require low levels of BR (Nakamura et al., 2003; Nemhauser et al., 2004; Hardke et al., 2007). Vert et al. (2008) recently showed that one mechanism by which BR signaling interacts with auxin signaling is by BR-mediated degradation of repressors of genes activated by auxin signaling. Additionally, the redundant transcription factors DORNRÖSCHEN/DORNRÖSCHEN-LIKE (DRN/DRNL) regulating e.g. embryo and lateral organ formation (Kirch et al., 2003) putatively act directly downstream of auxin responses (Chandler et al., 2007) and physically interact with BIM1 (BES interacting Myc-like protein 1; Chandler et al., 2009), a BR signaling associated transcriptional regulator binding to many BR-induces genes (Yin et al., 2005). This suggest that

BIM1 might contribute to DNA recognition and binding of DRN/DRNL to target promoters in certain situations (Chandler et al., 2009), thus integrating auxin and BR signaling by yet one more mechanism. BR has also been implicated to function in combination with ethylene and auxin in seedling development (De Grauwe et al., 2005).

Also CK and ABA effect LR formation, by repressing it (reviewed in Fukaki and Tasaka, 2008), and in addition CK, auxin and BR appear to interact in development of vascular tissues (reviewed in Dettmer et al., 2008). Auxin-mediated root branching is thus repressed by CK, while in the other side of the plant it is established that CK-mediated outgrowth of axillary buds is repressed by auxin (referred to as apical dominance; reviewed in Swarup et al., 2002) and removal of the auxin source in the aerial parts thus result in branching. Putatively, auxin regulates apical dominance in higher plants by repressing biosynthesis of CK in the node (Tanaka et al., 2006). CK and auxin also interact to control cell division by regulating components of the cell cycle (reviewed in Swarup et al., 2002).

1.2 IAA biosynthesis

IAA is mainly synthesized in rapidly growing and mitotically active tissues (Ljung et al., 2001a) by one or more of several different synthesis pathways. In *A. thaliana* at least three, putatively four, tryptophan (Trp)–dependent pathways, where Trp is the only established substrate, and at least one Trp-independent pathway where a Trp precursor is used as the primary substrate, are established to have functions in IAA biosynthesis (Figure 1; reviewed by Woodward and Bartel, 2005; Kriechbaumer et al., 2007; Delker et al., 2008; Chandler, 2009). Consequently, all auxin biosynthesis starts in the chloroplast where both Trp and its precursors are formed.

Trp-dependent and independent pathways have been suggested to take place in different sub-cellular compartments (Rapparini et al., 1999, 2002), however, enzymes have only been isolated and characterized in the Trp-dependent pathways. For Trp-dependent IAA biosynthesis Trp, or a Trp-related metabolite, depending on which Trp-dependent pathway is active, is exported to the cytoplasm where it is converted to IAA (reviewed in Cohen et al., 2003; Woodward and Bartel, 2005). IAA is thereafter used to induce signaling locally or it is transported from the site of biosynthesis to distant locations by polar auxin transport (PAT).

Redundancy of auxin biosynthetic pathways, both genetic with several genes exerting overlapping functions, and biochemical with several metabolites putatively acting as precursors for different IAA pathways,

complicate the dissection of what intermediates and enzymes are required for IAA biosynthesis in a given tissue under certain conditions, or by a specific environmental stimuli. The fact that several intermediates also are used in other metabolic pathways further complicate the picture.

The reason why such a wide array of auxin biosynthetic pathways exists is probably because the plant requires highly dynamic and strict control over production of auxin as a way to regulate growth and development in response to various genetic and environmental signals. The key to expand our understanding of these important processes is to map how the different pathways, ultimately leading to auxin, are regulated. In the following sections I will summarize what is known regarding biosynthesis of the most abundant naturally occurring auxin, IAA.

1.2.1 Tryptophan biosynthesis

The Shikimate pathway, ultimately producing chorismate, has been found only in plants, certain fungi and prokaryotes (reviewed in Hermann and Weaver, 1999). Production of chorismate in plants is localized to the chloroplast where it can be used as a precursor for production of the aromatic amino acids Trp, phenylalanine and tyrosine. Trp destined for IAA biosynthesis is either transported out of the chloroplast or first converted to Indole-3-acetaldioxime (IAOx) to the cytoplasm where it can be converted to IAA by several different pathways.

Six enzymes, or groups of enzymes, are required to create Trp from chorismate in *A. thaliana* (Figure 1). The first catalytic step, converts chorismate to anthranilate and is performed by ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1 or 2 (ASA1 or 2), or by ANTHRANILATE SYNTHASE BETA SUBUNIT 1 to 3 (ASB1 to 3; Radwanski and Last, 1995). Plant carrying mutations in *ASA1* and *ASB1* can be rescued by anthranilate, Trp and auxin treatment, suggesting that the products of these two genes eventually leads to the production of Trp that is mainly used by the plant for biosynthesis of IAA (Stepanova et al., 2005). Next, anthranilate is converted to 5-phospho-ribosylanthranilate by the single-copy gene product PHOSPHORIBOSYLANTHRANILATE TRANSFERASE 1 (PAT1; Rose et al., 1992) and subsequently to 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate by phosphoribosylanthranilate isomerases1 to 4 (PAI1 to 4; Bender and Fink, 1995). Thereafter, Indole-3-glycerol phosphate (IGP) is formed by INDOLE-3-GLYCEROL PHOSPATE SYNTHASE (IGPS; Ouyang et al., 2000). IGP can also be used as a substrate of the Trp-independent IAA biosynthesis, as will be described later. The two last steps are catalyzed by

TRYPTOPHAN SYNTHASE ALPHA SUBUNIT1 (TSA1) and TRYPTOPHAN SYNTHASE BETA SUBUNIT 1 and 2 (TSB1 and 2), creating indole and Trp, respectively (Figure 1; Normanly et al., 1993; Ouyang et al., 2000).

1.2.2 Trp-dependent IAA biosynthesis

The enzyme TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1), converting Trp to Indole-3-pyruvic acid (IPA), exert an important function, although not rate-limiting, in IAA synthesis via the IPA pathway, Trp -> IPA -> IAAld -> IAA (Figure 1; Stepanova et al., 2008; Tao et al., 2008). TAA1 has four homologues in *A. thaliana,* TRYPTOPHAN AMINOTRANSFERASE RELATED 1 to 4 (TAR1 to 4). TAA1, TAR2 and possibly TAR1 have overlapping functions, while the function of TAR3 and 4 remains elusive (Stepanova et al., 2008). An unknown enzyme converts IPA to IAAld. Interestingly, this conversion is catalyzed by a rate-limiting IPA decarboxylase in IAA-producing bacteria (Koga et al., 1994), suggesting that an IPA decarboxylase might have a similar role in plants (Tao et al., 2008). The last catalytic step of the IPA pathway has been suggested to be performed by aldehyd oxidase (AAO1; Seo et al., 1998).

In the two other established Trp-dependent pathways in *A. thaliana,* CYP79B2/3 and members of the YUCCA family of flavin monooxygenases, respectively, are suggested to act as rate-limiting enzymes (Zhao et al., 2001, 2002). These both pathways converge at the metabolite IAOx, which putatively is converted by unknown mechanisms to Indole-3-acetonitrile (IAN) or Indole-3-acetaldehyd (IAAld; Figure 1; Ljung et al., 2002).

In the YUC-pathway IAOx is produced by the conversion of Trp to tryptamine (TAM) by a putative tryptophan decarboxylase (TDC) and to N-hydroxyl-TAM by the YUC members (Zhao et al., 2001). The mechanism underlying the formation of IAOx from N-hydroxyl-TAM remains unknown. CYP79B2/3 on the other hand directly converts Trp to IAOx in a single catalytic reaction (Hull et al., 2000).

The enzyme catalyzing the putative formation of IAOx to IAN is as previously mentioned unknown, but *A. thaliana* members of the *Brassicaceae*-specific nitrilase group, consisting of NIT1 to 3, have been shown to catalyze the next step of this pathway, the formation of IAA from IAN, although with a very low efficiency (Vorwerk et al., 2001). NIT1 to 3 show much higher affinity for other substrates not implicated to function in IAA biosynthesis (Vorwerk et al., 2001). However, if the substrates are

compartmentalized, or if there is a coupled enzymatic reaction where the different enzymes in the pathway are physically connected, in a way making IAN the only substrate available in large quantities, NIT1 to 3 are thought to efficiently catalyze IAA from IAN (Vorwerk et al., 2001). NIT homologues from other species, e.g. maize (*Zea mays*), can also convert IAN to IAA (Park et al., 2003). The maize NIT2 enzyme hydrolyzes IAN to IAA with an efficiency at least 7- to 20-fold higher than for *A. thaliana* NIT1/2/3 enzymes. The maize NIT2 protein, as well as all NIT proteins from species outside the *Brassicaceae*, is more similar in sequence to the *A. thaliana* NIT4 protein, than to NIT1/2/3. Interestingly, NIT4 is the only NIT in *A. thaliana* not able to catalyze IAN to IAA *in vitro* (Vorwerk et al., 2001; Park et al., 2003).

There is also putatively a fourth Trp-dependent pathway in plants. This pathway, named after the metabolite indoleacetamide (IAM), is known previously from prokaryotes (Weiler and Schroeder, 1987). Although IAM has been isolated from plant extracts (Kawaguchi et al., 1991, 1993; Pollmann et al., 2002), the enzyme producing this intermediate has not been characterized. However, it has been reported that nitrilases in the cytoplasm putatively can catalyze the reaction from Trp to IAM, indicating a second function for nitrilases in IAA biosynthesis (Pollmann et al., 2002). Further evidence for an IAM pathway in *A. thaliana* came when Pollmann et al. (2003, 2006) and Neu et al. (2007) showed that the cytoplasmic enzyme AMIDASE1 (AMI1) can catalyze IAM to IAA *in vitro*, and that expression of *AMI1,* as well as abundance of the AMI1 protein, peaks in regions known as high auxin zones e.g. young leaves. Although the *in vivo* function of AMI1 remains to be characterized, this pathway might deserve to be seriously considered also in plants.

1.2.3 Trp-independent IAA biosynthesis

The last auxin biosynthetic pathway to be mentioned here bypass Trp by originating from the Trp-precursor IGP (Ouyang et al., 2000), and has thus been named the Trp-independent pathway (Normanly et al., 1993; Wright et al., 1991; Figure 1). The existence of this pathway is suggested by the fact that loss-of-function mutations in *PAT1*, but not in *TSA1* or *TSB1*, totally block formation of IAA conjugates (Last and Fink, 1988), implicating that IAA can be synthesized independent of Trp. Additionally, antisense plants with reduced IGPS levels, do not accumulate IAA conjugates, further establishing IGP as the branch point between Trp-independent and Trp-dependent pathways (Normanly et al., 1993; Ouyang et al., 2000).

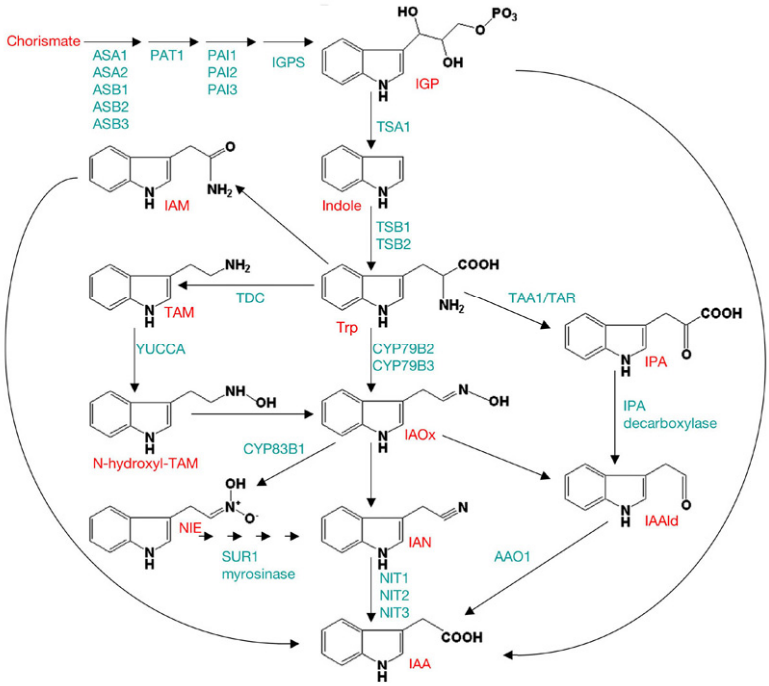

Figure 1. IAA biosynthesis pathways in *A. thaliana*. Adapted from Woodward and Bartel, (2005) and Tao et al. (2008). Red indicates metabolites; Blue indicates enzymes. Abbreviations: AAO, ARABIDOPSIS ALDEHYDE OXIDASE; ASA, ANTHRANILATE SYNTHASE ALPHA SUBUNIT; ASB, ANTHRANILATE SYNTHASE BETA SUBUNIT; IAA, Indole-3-acetic acid; IAAld, Indole-3-acetaldehyd; IAM, Indoleacetamide; IAN, Indole-3-acetonitrile; IAOx, Indole-3-acetaldioxime; IGP, Indole-3-glycerol phosphate; IGPS, INDOLE-3-GLYCEROL PHOSPATE SYNTHASE; IPA, Indole-3-pyruvic acid; NIE, 1-aci-Nitro-2-Indolylethane; NIT, NITRILASE; PAI, PHOSPHORIBOSYL ANTHRANILATE ISOMERASE; PAT, PHOSPHORIBOSYL ANTHRANILATE TRANSFERASE; SUR, SUPER ROOT; TAA, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS; TAM, Tryptamine; TAR, TAA1 RELATED; TDC, TRYPTOPHAN DECARBOXYLASE; Trp, Tryptophan; TSA, TRYPTOPHAN SYNTHASE ALPHA SUBUNIT; TSB, TRYPTOPHAN SYNTHASE BETA SUBUNIT;.

Not much is known of the different enzymes downstream of IGPS in the Trp-independent pathway. Nevertheless, this is considered an important IAA biosynthetic pathway for the regulation of plant growth and development because of several reasons (reviewed in Ljung et al., 2002), e.g. intact *A. thaliana* seedlings cannot efficiently convert Trp into IAA, however, the Trp precursor anthranilate is efficiently converted to IAA (Normanly et al., 1993). *A. thaliana* shoot and root explants can however efficiently convert Trp to IAA (Müller et al., 1998; Müller and Weiler,

2000), indicating that different IAA biosynthetic pathways operate under different physiological conditions. Additionally, several studies on diverse species show that the Trp-independent pathway contributes to more than 75% of the total amount of IAA in certain tissues (Baldi et al., 1991; Sitbon et al., 2000; Wright et al., 1991).

1.3 Integration of biosynthetic pathways for production of IAA and defense related secondary metabolites

The YUC and CYP79B2/3 Trp-dependent pathways in *A. thaliana* may be closely integrated with biosynthesis of indolyl glucosinolates (IG) and camalexin, as IAOx is a common intermediate for these three pathways (Figure 2; Grubb and Abel, 2006). Both IGs and camalexins are associated with pathogen response signaling in *A. thaliana*. Camalexin is used by the plant as an antimicrobial compound (Paxton 1981) and IG, which is a *Brassicaceae*-specific compound, is utilized as a precursor for toxic secondary metabolites (reviewed in Kliebenstein et al., 2005). Additionally, *in vivo* feeding experiments showed that the precursor IAOx can act as a precursor for other indole-derived compounds (Pedras et al., 2001), suggesting that the different pathways for synthesis of primary and secondary metabolites from IAOx must be compartmentalized to avoid intermixing, and/or that the enzymes downstream of IAOx are strictly regulated at the transcriptional or post-transcriptional level.

IAOx can also be shunted to biosynthesis of IG via the enzyme CYP83B1 (Bak and Feyereisen, 2001), converting IAOx to the IG precursor 1-aci-Nitro-2-Indolylethane (NIE; Figure 1), or to biosynthesis of camalexin via CYP71A13 (Nafisi et al., 2007), catalyzing the reaction of IAOx to IAN. The latter reaction is thought to be cytoplasmic and would imply yet another common intermediate between IAA and camalexin synthesis pathways, in addition to IAOx (Nafisi et al., 2007). As previously mentioned, the enzyme catalyzing the conversion of IAOx to IAN in the IAA biosynthesis pathway is unknown. However, CYP71A13 and/or related enzymes could be putative candidates, although this remains to be established. Myrosinases, usually associated with pathogen attack responses, can putatively produce IAN by degrading IG. This pathway, using IG as a source for IAA mobilization, has been established, although its physiological relevance remains to be confirmed (Pollmann et al., 2002; Vorwerk et al., 2001; Kutz et al., 2002).

IAA levels are increased, and IG levels reduced, in the *CYP83B1* loss-of-function mutants (*sur2/atr4/red1*), suggesting that IAOx not used for IG

metabolism is used for biosynthesis of IAA (Bak and Feyereisen, 2001; Delarue et al., 1998; Barlier et al., 2000; Smolen and Bender, 2002; Hoecker et al., 2004). Because the *cyp79b2* and *cyp79b3* mutants have reduced IG levels, and the *cyp79b2 cyp79b3* double mutant completely lacks IG and camalexin, and only displays reduced IAA biosynthesis rates (Zhao et al., 2002; Ljung et al., 2005; Glawischnig et al., 2004) it has been suggested that the main function of CYP79B2/3 is in IG/camalexin and not IAA, biosynthesis. An alternative explanation is that all IG and camalexin production is channeled by CYP79B2/3, making them essential for production of these compounds, while there are several parallel and partially redundant IAA biosynthesis pathways, making the CYP79B2/3 contribution less significant for the total IAA pool.

YUC overexpressor lines show high IAA but low IG levels, suggesting that the cytoplasmic IAOx produced by YUC never is used for IG production and hence different IAOx pools for IAA and IG/camalexin biosynthesis are likely to exist. CYP79B2/3 most likely convert Trp to IAOx in the chloroplast (Hull et al., 2000; Ljung et al., 2002), from where IAOx may be transported to the ER, where it may act as a substrate for CYP83B1 and CYP71A13, both suggested to be localized at the cytoplasmic side of the ER membrane (Cohen et al., 2003). This indicates that the cellular transport and compartmentalization of IAOx could be a major control node for separating these pathways. Additionally, coupled enzymatic reactions, where the different enzymes in a pathway are physically connected, thus forcing the intermediate substrates/products to never leave the enzymatic complex, also could provide an explanation for why IAOx produced for IAA synthesis is not a substrate for IG/camalexin synthesis (Abel and Grubb, 2006). This explanation is also likely for the YUC-pathway-mediated Trp to IAOx conversion since TAM is hard to detect in plants (Ljung et al., 2002), suggesting that it is metabolized very quickly.

As mentioned, the YUC family of flavine monooxygenases catalyzes the formation of N-hydroxyl-TAM from TAM (Zhao et al., 2001; Figure 1). Interestingly, N-hydroxyl-TAM has been shown to bind tightly to the active site of CYP83B1 and inhibit its catalysis (Bak et al., 2001; Bak and Feyereisen, 2001). This could be a mechanism just as important as compartmentalization, or coupled enzymatic reactions, for resolving to which pathway newly synthesized IAOx will go.

These findings suggest that IAOx produced by the YUC pathway specifically is channeled to IAA biosynthesis and not to production of IG (Grubb and Abel, 2006). It has also been shown that CYP79B2/3 is induced by pathogens, wounding and MeJA (stress signaling hormone), which

verifies that one biological function of CYP792/3 indeed is to produce defense-related secondary metabolites in response to pathogen attack (Mikkelsen et al., 2003; Glawischnig et al., 2004). Additionally, no CYP79B2/3 homologues have been identified in rice, a plant without IG biosynthesis, suggesting that the YUC pathway produce all IAOx for IAA biosynthesis in this plant (Yamamoto et al., 2007). It thus appears that CYP79B2/3 has dual biological functions in *A. thaliana*, while YUC and TAA1/TAR2, so far, only have established functions in IAA biosynthesis.

Taken together, the YUC and CYP79B2/3 IAA biosynthesis pathways are closely integrated with other pathways producing indole-related compounds. However, it is possible that the pathways are separated in time and in space, both at a higher level (cell types, tissues and organs), but also in the sub-cellular space. The fact that many of the reactions take place in the same sub-cellular compartment, the cytoplasm, suggest that activity of the different enzymes is strictly regulated, most likely including regulation at the transcriptional level and possibly by further compartmentalization within the cytoplasm, e.g. in enzymatic/catalytic complexes.

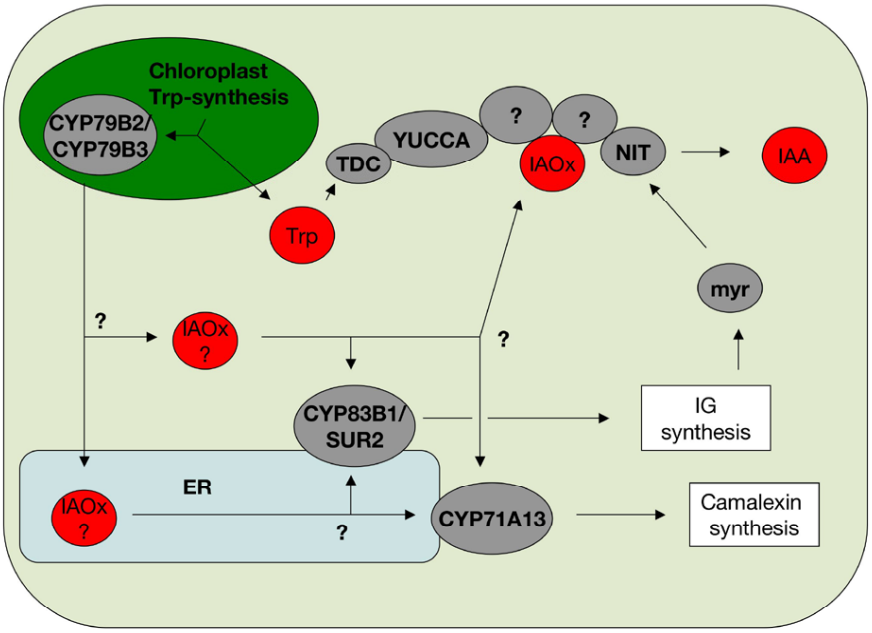

Figure 2. Putative cellular compartmentalization of YUC-, CYP79B2/3- and CYP71A13-mediated IAOx, IAA, IG and camalexin biosynthesis. Green, the chloroplast; blue, the endoplasmic reticulum (ER); grey, proteins; red, Trp and Trp-derived metamolites; myr, myrosinase. Other abbreviations are explained in the text.

1.4 Implications of divergent roles of different IAA biosynthesis pathways in plant development

Recent data suggest that different auxin biosynthesis pathways may actually operate in different tissues under different physiological circumstances.

1.4.1 The IPA pathway

The *TAA1* loss-of-function mutants, known as *weak ethylene insensitive8 (wei8)* or *shade avoidance3* (*sav3),* were simultaneously characterized using different approaches (Tao et al., 2008; Stepanova et al., 2008).

Several *wei* mutants were identified in a screen for weak ethylene insensitivity (Alonso et al., 2003). Of the identified genes, *WEI2/ASA1* and *WEI7/ASB1,* are associated with Trp synthesis, and *WEI8* is allelic to *TAA1* (Figure 1; Stepanova et al., 2005; Stepanova et al., 2008). In the parallel study, Tao et al. (2008) searched for mutants unable to elongate under low R/FR light, simulating shade, and identified *sav3,* also allelic to *TAA1.* The IAA levels are reduced in the *sav3* and *wei8* mutants, and the *sav3* mutant has lost the ability to respond to shade with increased IAA biosynthesis rates (Tao et al., 2008), suggesting an impaired auxin biosynthesis.

TAA1 is expressed in root tips, cotyledon margins, young leaves and in floral organs (Stepanova et al., 2008; Tao et al., 2008). The *TAA1* homologue *TAR2* is also expressed in these tissues, although the expression patterns do not fully overlap. Accordingly, the *wei8 tar2* double mutants show defects in root and floral organ formation, with severe defects in e.g. the gynoecia (Stepanova et al., 2008). The expression pattern of *TAR1* is not fully established but appear to deviate from *TAA1/TAR2* (Stepanova et al., 2008).

The distinct expression patterns suggest that auxin produced by the IPA pathway is transported over long distances to various sites, e.g. the hypocotyls, where IAA induce cell elongation and thus shade avoidance (Tao et al., 2008), but that it also can be used locally, e.g. in the root tip or the flower, to modulate growth (Stepanova et al., 2008). However, the absence of an auxin-overproduction phenotype in the *TAA1* overexpressor (Tao et al., 2008) suggest that another factor acts rate-limiting for auxin synthesis in this pathway.

Taken together, mutant and expression analyses of *TAA1* and *TAR2* reveal cross-talk between tissue specific ethylene action and auxin production and demonstrate the importance of quickly induced auxin biosynthesis in growth responses to environmental factors i.e. light quality.

1.4.2 The CYP79B2/3 pathway

Light-grown *CYP79B2* overexpressor lines are resistant to toxic Trp analogues and have long hypocotyls and epinastic leaves, phenotypes resembling those of *sur2* or overexpressor of the bacterial IAA biosynthesis gene *IaaM* (Klee et al., 1987; Barlier et al., 2000; Zhao et al., 2002), suggesting that CYP79B2 overexpressor plants are auxin overproducers.

CYP79B2 is expressed in the cotyledons, roots, the style/stigma and abscission zone of the gynoecium and silique, respectively, and in the vasculature of most organs (Mikkelsen et al., 2000). *cyp79b2* and *cyp79b3* single mutants show no visible defects however the *cyp79b2 cyp79b3* double mutant have reduced petiols and smaller leaves, is hypersensitive to toxic Trp analogues and at high temperatures *cyp79b2 cyp79b3* seedlings produces less IAA than wild type seedlings (Zhao et al., 2002). Additionally, the adult plant has even smaller and slightly curved leaves, resembling auxin-resistant mutants (Zhao et al., 2002). Interestingly, auxin biosynthesis in hypocotyls is increased at high temperatures, supporting a function of *CYP79B2/3* in auxin biosynthesis (Gray et al., 1998).

The expression patterns, auxin measurements and phenotypes in mutants and overexpressors suggests *CYP79B2/3* to have a function in auxin biosynthesis but interestingly, elimination of the CYP79B2/3 pathway only gives minor developmental defects, suggesting that other pathways act redundantly.

1.4.3 The YUC pathway

YUC1 was identified as an activation tagging line (*yucD*) displaying elongated hypocotyls and epinastic cotyledons when grown in light, and short hypocotyls and the lack of an apical hook when grown in dark. The *yucD* line also display a short and hairy root and increased apical dominance and is resistant to toxic Trp analogues, much like the $35S_{pro}$*:CYP79B2* or the $35S_{pro}$*:IaaM* lines (Klee et al., 1987; Zhao et al., 2001, 2002). There are 11 *YUC* genes in *A. thaliana* (Cheng et al., 2006) and all analyzed *YUC* overexpressor lines show increased auxin levels and identical phenotypes suggesting redundant functions (Zhao et al., 2001; Cheng et al., 2006; Kim et al., 2007). Indeed, *YUC1, 4, 10* and *11* have overlapping expression patterns in the apical region of the globular embryo and later in the cotyledon primordia and apical region (Cheng et al., 2007). In the mature embryo expression is mainly restricted to the apical region (Cheng et al., 2007). Unlike the other examined genes *YUC4* is also expressed in the cotyledon tips of mature embryos (Cheng et al., 2007). However, no embryonic defects could be detected in the *yuc* mutants, including the *yuc1*

yuc4 double mutant, most likely a result of the high level of redundancy (Cheng et al., 2007). *YUC1* and *4* expression in the seedling is restricted to cells flanking the shoot apex, to stipules and to the base of newly formed leaves and *YUC4* is additionally expressed throughout young leaf primordia and in the apical parts of young leaves (Cheng et al., 2007). The *yuc1 yuc4* mutant show reduced $DR5_{pro}$*:GUS* signal in seedlings and slightly defective vasculature and the *yuc1 yuc2 yuc4 yuc6* quadruple mutants display abnormal leaves, suggesting redundancy also in leaf formation (Cheng et al., 2006, 2007).

YUC1, 2, 4 and *6* are also expressed to varying degrees in partially overlapping domains of the inflorescence apex and floral organs (Cheng et al., 2006). Expression is found e.g. in the base of developing organs (*YUC4*), in the style of young (*YUC2, 4*) and mature gynoecia (*YUC4*), in stamens (*YUC2, 4, 6*), in pollen (*YUC6*) and in petals (*YUC2*). These slight variations in expression pattern reveal some functional differences between YUC members in flower development. The *yuc1 yuc4* mutant displays defects in all floral organs and have no functional reproductive organs, while the *yuc2 yuc6* mutant only have short stamens and defective and mostly sterile pollen. The quadruple mutant has a small inflorescence meristem and rarely produces buds, however the buds that are formed display a severely disturbed morphology (Cheng et al., 2006). Interestingly, all developmental defects of the *yuc1 yuc4* double mutant can be rescued by *YUC1* promoter driven expression of the auxin biosynthesis gene *IaaM* (Cheng et al., 2006), suggesting that the defects are caused by reduced levels of active auxin.

Thus, *YUC* members have established functions in genetically regulated development, e.g. organ initiation and morphogenesis (Cheng et al., 2006, 2007; Kim et al., 2007), and so far no connections to environmental regulations have been made. Instead, it appears as if *YUC* and *TAA1/TAR2* at large, act non-redundantly in different developmental programs.

1.5 Transcriptional regulation of IAA biosynthesis

It appears as if the different biosynthesis pathways to a high degree take part in different physiological and developmental programs, thus suggesting that the rate-limiting enzymes are differentially regulated.

The genes encoding the suggested rate-limiting enzymes CYP79B2 and YUC members, as well as the TAA1/TAR2 enzymes are probable targets for a high degree of transcriptional regulation. However, virtually nothing was known about proteins acting as transcriptional regulators of auxin biosynthesis, until recently (reviewed in Chandler, 2009).

Sohlberg et al. (2006) reported that the putative transcription factor STYLISH1 (STY1) activate transcription of *YUC4* in floral buds and in seedlings, and that induction of ectopic STY1 activity increase IAA levels in seedlings. Interestingly, mutations in *STY1* and related genes affect the levels of several IAA precursors, e.g. IAN levels are reduced by 50% in a *SHI/STY* quintuple mutant (Sohlberg et al., 2006). The effect of STY1 on auxin biosynthesis was later confirmed when Ståldal et al., (2008) could show that induction of STY1 results in increased IAA biosynthesis rates. The establishment of STY1 as a DNA-binding transcriptional activator regulating expression of enzymes probably rate-limiting for IAA biosynthesis is discussed in the results section (chapters 3.5 and 3.6).

Additionally, LEAFY COTYLEDON2 (LEC2; Meinke et al., 1994; Stone et al., 2001) was recently shown to directly regulate auxin biosynthesis related genes (Stone et al., 2008). LEC2 is a B3 domain transcription factor regulating several aspects of embryogenesis (Meinke et al., 1994; Stone et al., 2001). Loss-of-function mutations in *LEC2* have e.g. disrupted maintenance of embryonic cell fate and specification of cotyledon identity, suggesting that it is active during the early events of embryogenesis, in which the basic body plan of the embryo is established (Stone et al., 2001). These phenotypic abnormalities are likely an effect of reduced auxin levels because convincing evidence, e.g. chromatin immunoprecipitation (ChIP), reporter constructs and transcript measurements, suggests LEC2 to directly bind and activate the promoters of *YUC4* and *YUC6*, resulting in increased auxin signaling (Stone et al., 2008).

The third transcription factor reported to function in regulation of auxin biosynthesis is SPOROCYTELESS (SPL; Li et al., 2008). SPL regulates lateral organ morphogenesis (Li et al., 2008) and is associated to sporocyte formation (Yang et al., 1999). Constitutive expression of *SPL* negatively modulates expression of *YUC2* and *YUC6* in leaves and flowers, and therefore its effects on lateral organ shape was connected to reduced auxin levels (Li et al., 2008). However, *SPL* is not expressed in detectable amounts in the leaves and the effects on auxin biosynthesis and auxin mediated leaf development is only seen in the SPL overexpressor, thus no abnormalities are found in leaves of the loss-of-function mutant *spl* (Li et al., 2008). Additionally, SPL has not yet been shown to bind the promoters of *YUC2* and *YUC6* (Li et al., 2008) and therefore a function of SPL as a direct transcriptional regulator of auxin biosynthesis remains uncertain (Li et al., 2008).

To my knowledge, these are the only transcription factors shown to regulate auxin biosynthesis, and thus much remain to be done in order to

understand how different auxin biosynthesis pathways are regulated at the transcriptional level.

1.6 Auxin transport

Polar auxin transport (PAT) in plant tissues has been known for more than 80 years and is fundamental for plant growth and development (reviewed in Friml, 2003). The mediators of this trans-cellular auxin transport are transmembrane proteins that function as either efflux or influx facilitators. These facilitates the uptake and release of auxin from/to the inter-cellular space, the apoplast (reviewed in Teale et al., 2006; Delker et al., 2008; Kramer and Bennett, 2006; Vieten et al., 2007; Benjamins and Scheres, 2008).

Many auxin transport facilitators are well characterized: The AUXIN PERMEASE1/LIKE AUXIN PERMEASE (AUX1/LAX) protein family performing auxin influx (Bennet et al., 1996), the PIN-FORMED (PIN) family of auxin efflux facilitators (Blilou et al., 2005), and several MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (MDR/PGP) ABC transporters (Geisler and Murphy, 2006), e.g. MDR1 mediating acropetal transport (Terasaka et al., 2005) or MDR4/PGP4 mediating basipetal transport (Lewis et al., 2007).

The dynamic expression of these proteins and their polar localization in the plasma membrane define the direction of PAT throughout the life of the plant and mutations in the above mentioned genes frequently results in very severe developmental abnormalities. The *pin1* loss-of-function mutant displays, as the name imply, a naked stem with no lateral organs, there are however weak *pin1* alleles morphologically similar to wild type plants (Bennett et al., 1995). The weak *pin1* allele *pin1-5* in *sty1* mutant background suggests that *PIN1* and *STY1* affect gynoecium development synergistically (Sohlberg et al., 2006). Additionally, the *pin1-5 sty1* mutant resemble NPA treated *sty1 sty2* mutant gynoecia (Sohlberg et al., 2006), establishing a connection between regulation of *YUC* members and PAT in gynoecium morphogenesis. A *pin1-5 yuc1 yuc4* triple mutant was later shown to phenocopy the strong *pin1* allele (Cheng et al., 2007), demonstrating the requirement for both IAA synthesis and transport in *YUC*-mediated regulation of organ initiation.

In addition to PAT, auxin can also be transported (e.g. from the leaves to the roots) in the phloem in where auxin is loaded to/from the vasculature by AUX1 (Marchant et al., 1999, 2002; Swarup et al., 2001). Auxin can also diffuse from the apoplast, over the plasma membrane, into the cytoplasm.

This passive influx requires that IAA is protonated, which it is in the acidic apoplast, however once in the cytoplasm (pH 7) IAA gets deprotonated and thus cannot diffuse out of the cell (reviewed in Kramer and Bennet, 2006). The latter influx mechanism is probably important, however, the AUX1-mediated influx has been shown to be ten times as efficient in some situations (Yamamoto and Yamamoto, 1998) and loss-of-function mutations in *AUX1* results in e.g. reduced gravitropism and reduced lateral root formation (Marchant et al., 1999, 2002).

1.7 Auxin degradation and homeostasis

Plant maintenance of correct cellular auxin levels requires biosynthesis and transport, as already described. However, plants also store and transport auxin as inactive conjugates and in certain physiological conditions, e.g. during early seedling growth in Scots pine (*Pinus sylvestris*; Ljung et al., 2001b), the previously mentioned mechanisms appear to be less important for the total pool of IAA as most active IAA is formed by hydrolysis of a pool of stored IAA conjugates (reviewed in Ljung et al., 2002; Woodward and Bartel, 2005). The conjugation of IAA is also a way for the plant to reduce the levels of free active IAA in response to excessive auxin levels. This reduction is achieved e.g. by the auxin-mediated induction of several GH3 family members capable of conjugating IAA to amino acids (Figure 3; Hagen and Guilfoyle, 1985; Östin et al., 1998; Staswick et al., 2005), thus functioning as a negative feed-back loop of auxin responses. Conjugation and its subsequent catabolism is also a way for the plant to degrade IAA, permanently removing it from the system. The mechanism chosen by the plant to degrade or inactivate auxin thus depend on the developmental stage and physiological state.

Members of the GH3 family of amide conjugating enzymes catalyze the formation of IAA conjugated via amide bonds to Ala, Leu, Asp and Glu (Figure 3; reviewed in Ljung et al., 2002; Staswick et al., 2005). The IAAla and IALeu conjugates can be hydrolyzed to produce IAA by hydrolytic enzymes e.g. IAR3 (Davies et al., 1999) and ILR1 (Bartel and Fink, 1995), respectively. The IAAsp and IAGlu conjugates are however irreversible and leads to IAA catabolism (Östin et al., 1998).

IAA can also be attached to sugars via ester-bonds by e.g. UDP glucose transferases (UGT), resulting in the formation of IAA-glucose conjugates (Szerszen et al., 1994; Jackson et al., 2001). These conjugates are hydrolyzed by IAA-glucose hydrolases (reviewed in Woodward and Bartel, 2005).

There are also other forms of conjugates than those mentioned here, e.g. protein or peptide conjugates (Seidel et al., 2006).

In addition to auxin degradation by conjugation, the plant can catabolize IAA to the terminally inactive form oxIAA by the action of an IAA oxidase (Reinecke and Bandurski, 1987), or to other terminally inactive forms by various plant-specific pathways, ultimately resulting in oxidation of IAA (reviewed in Ljung et al., 2002).

Other ways to convert IAA into other inactive or less active forms is the conversion of IAA to IBA by an IBA syntase (reviewed in Woodward and Bartel, 2005) and the methylation of IAA by an auxin carboxyl methyltransferase (IAMT1; Zubieta et al., 2003; Qin et al., 2005). Although overexpression of *IAMT1* leads to hyponastic leaves and RNAi-mediated reduction of *IAMT1* transcript levels leads to epinastic leaves, the function of MeIAA is unclear. The leaf phenotypes however strongly suggest MeIAA to be an inactive auxin important for auxin homeostasis (Qin et al., 2005).

These mechanisms of inactivation, conjugation and hydrolysis is of major importance for auxin homeostasis in *A. thaliana* and other higher plants while its importance in lower plants and specifically in algae might be diminished, in favor for auxin biosynthesis (Cooke et al., 2002) as will be described later.

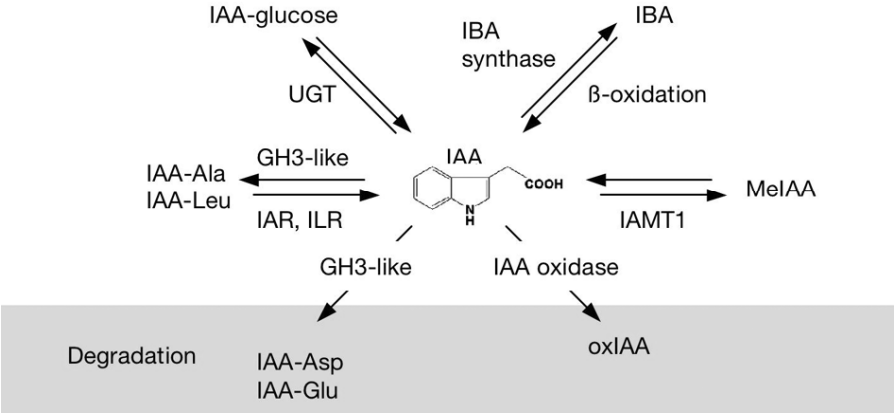

Figure 3. Simplified view of IAA storage and degradation pathways in *A. thaliana*. Adapted from Chandler (2009).

1.8 Auxin perception and signaling

In plants there are two groups of known, and at least partially characterized, auxin receptors. The most studied receptor is TRANSPORT INHIBITOR RESPONSE1 (TIR1; Kepinski and Leyser, 2005; Dharmasiri et al., 2005a) and its homologues in *A. thaliana* AUXIN SIGNALING F-BOX PROTEIN1 to 3 (AFB1 to 3; Dharmasiri et al., 2005b). In addition to this family of well characterized IAA receptors there is AUXIN BINDING PROTEIN1 (ABP1) for which auxin binding capacity was described already by Hertel et al. (1972). In *A. thaliana ABP1* is a single-copy gene and the protein product was for a long time the only candidate for an auxin receptor (Jones, 1994). Because of its embryo lethality the *abp1* loss-of-function mutant has been difficult to study, and thus the function of *ABP1* in the adult plant has not been properly established (Chen et al., 2001). It is known that ABP1 binds auxin at physiologically relevant levels, that it can discriminate between active and inactive forms of auxin and that it mediates ion-fluxes across the plasma membrane in response to auxin treatment, subsequently resulting in cell-expansion (Venis et al., 1992; Rück et al., 1993; Thiel et al., 1993; Leblanc et al., 1999; Bauly et al., 2000). This provides an explanation to the observation that e.g. the hypocotyls elongate when subjected to physiologically relevant levels of auxin. Recently, David et al. (2007) and Braun et al. (2008) could show, using conditional repression of *ABP1* in adult plants, that ABP1 coordinates cell-division and cell-expansion during postembryonic shoot development in *A. thaliana* and Tobacco. However, the downstream signaling targets and molecular function of ABP1 remain to be properly established.

On the other hand, the molecular function of TIR1/AFB family members is well studied (Kepinski and Leyser, 2005; Dharmasiri et al., 2005a, b). *TIR1/AFB1* to *3* encode F-box proteins part of an SKP1/CULLIN/F-BOX PROTEIN (SCF) complex ($SCF^{TIR1/AFB1/2/3}$), also containing the proteins ASK1, CUL, RBX1 and an E2 ligase in addition to the TIR1/AFB receptors (Figure 4), that mediates the ubiquitination, and thereby 26S proteasome-dependent degradation, of targeted proteins (reviewed in Abel et al., 2007). The finding that the TIR1/AFB proteins act as auxin receptors was strengthened by the fact that protein turn-over via an SCF complex already was an established feature of auxin signaling (Gray et al., 1999, 2001; Quint and Gray, 2006).

Auxin binding to a hydrophobic pocket within the TIR1 protein (Tan et al., 2007) stabilize the interaction between TIR1 and AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins, thereby targeting the AUX/IAAs, already having very high turn-over rates, for degradation (Abel et al., 1994;

Zenser et al., 2001),. The function of AUX/IAA proteins is to inactivate auxin responses by forming dimers with DNA-binding auxin response factors (ARFs), thus acting as repressors of gene expression. Mutations in the AUX/IAA domain recognized by TIR1/AFB1 to 3 result in stabilization of the proteins and thus to developmental defects caused by reduced auxin sensitivity (reviewed in Benjamins and Scheres, 2008). The IAA/TIR1-complex-mediated degradation, which removes the AUX/IAA proteins from the ARFs, allow the ARFs, acting a DNA-binding transcriptional activators or repressors, to bind to auxin responsive elements (AuxREs), typically represented by the sequence TGTCTC (Ulmasov et al., 1997a, 1999). This interaction modulates transcription of the targeted genes, subsequently leading to alteration of developmental programs (Figure 4. Quint and Gray, 2006; Benjamins and Scheres, 2008).

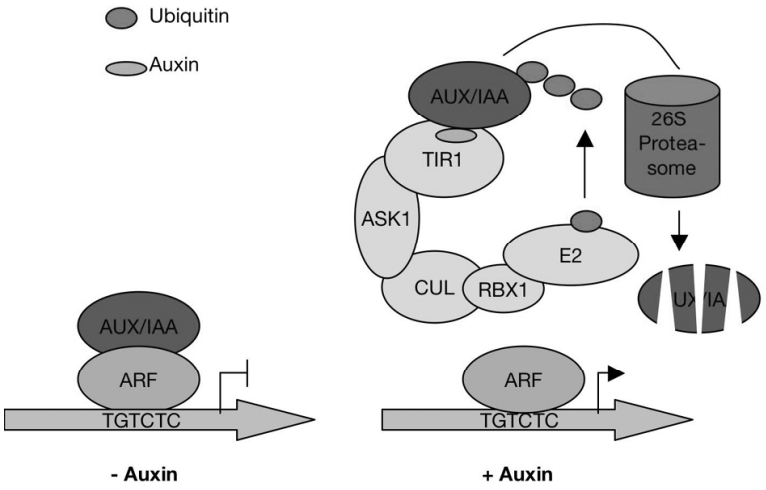

Figure 4. Simplified model of auxin-mediated transcriptional regulation via the TIR1/AFB auxin receptors. ASK1, ARABIDOPSIS SKP1-LIKE; CUL, CULLIN; E2, a conjugating enzyme (E2-ligase); RBX1, RING-BOX PROTEIN1. TGTCTC is an AuxRE.

Auxin responses thus include transcriptional induction of several gene families, e.g. *SMALL AUXIN-UP RNAs* (*SAUR*), *GRETCHEN HAGEN3* (*GH3*) and *AUX/IAA* (reviewed in Woodward and Bartel, 2005). The auxin-dependent induction of these genes can be detected within minutes after auxin application, e.g. *GH3* expression can be detected already 5 min after auxin application (Hagen and Guilfoyle, 1985). Hence, these gene families were classified as primary response genes of auxin action. It is believed that *AUX/IAAs* are up-regulated by auxin as a feed-back mechanism to prevent negative action of too high auxin responses (Abel et

al., 1994). This is thus a mechanism working in parallel to the GH3 proteins, which as previously mentioned, act to catalyze the formation of inactive IAA conjugates thereby reducing the level of free, active, IAA. *SAUR* transcripts are short lived and the proteins have high turn-over rates, their function however remain elusive (Walker and Key, 1982).

The model summarized in Figure 4 does however not explain the function of those ARFs acting as transcriptional repressors. However, a recent report by Vert et al. (2008) provides evidence of a BR-mediated induction of gene expression via repressor ARFs. Vert et al. (2008) propose that repressor ARFs, i.e. ARF2, can compete for binding to AuxREs with activator ARFs. BR-mediated degradation of ARF2 would result in removal of ARF2 from the promoter and thus to loss of ARF2 repression activity. This would then lead to the activation of the targeted gene by another ARF in the presence of auxin. BR thus removes the repressor while IAA activates the activator. Indeed, it has been shown that some auxin induced genes and synthetic auxin signaling markers, e.g. *DR5pro:GUS* (Ulmasov et al., 1997b) are fully activated only in the presence of both auxin and BR (Nakamura et al., 2003; Nemhauser et al., 2004).

In conclusion, TIR1/AFB-mediated auxin responses are restricted to direct modulation of gene expression by the degradation of proteins acting as transcriptional repressors, or repressors of ARF function, while the function of ABP1 include induction of cell-division and signaling over the plasma membrane to induce cell-expansion.

1.9 *Physcomitrella patens* as a model organism

The moss *Physcomitrella patens* is a small, up to 5 mm high, plant of the family *Funariaceae* (order *Funariales*) comprising about 300 species (Buck and Goffinet, 2000) found all over the world, and is believed to be reminiscent of the first plants that moved from water to land, the ancestral embryophytes.

Mosses, together with liverworts and hornworts, constitute the bryophytes that diverged from the rest of the land plants some 450 million years ago (Rensing et al., 2008). The bryophytes resemble the ancestral land plants by being gametophyte dominant with a sub-ordinate sporophyte generation. Thus, the dominant generation in bryophyte development is haploid. Most available data suggest that evolution of the diploid sporophyte dominating higher plants today involved the recruitment of already existing gametophytic developmental programs (Floyd and Bowman, 2007; Menand et al., 2007). To define the basis from which developmental programs in

higher plants have evolved, comparative studies have emerged as an important tool, since seemingly similar traits, existing in two different organisms, can generally be expected to have originated in their common ancestor.

The members of the bryophyte lineage are quite diverse in several aspects but have common features separating them from other plant groups (Raven et al., 1992). As mentioned, all bryophytes have a dominant haploid generation. They also generally lack true vasculature, although some genera have specialized conductive cells/tissues. Also, bryophytes do not have roots, instead they use rhizoids to attach themselves to their place of growth. Otherwise, many basic and fundamental genetic and physiological processes in the haploid generation of bryophytes appear to be shared with the diploid sporophyte generation of vascular plants.

Mosses in general were recognized as possible models for plant development more than 50 years ago (Barthelmess, 1939, 1940; von Wettstein, 1925). The foremost reasons for using moss as a model organism is the simple morphology (Cove, 2005; Cove et al., 1991, 2006), and as described above, the evolutionary position. Another reason being that the dominant stage of the life cycle is haploid, enabling studies of recessive mutations without backcrossing to generate homozygous diploids, dramatically reducing the time from transformation to phenotypic characterization.

The foremost reason why *P. patens* in later years has become the major bryophyte model is that it is the only identified plant in which gene targeting, via homologous recombination, can be performed efficiently (Schaefer and Zrüd, 1997; Shaefer, 1991). In fact, gene targeting in *P. patens* is almost as efficient as in yeast, i.e. five orders of magnitude higher than for other plants (Reski and Cove, 2004). However, Trouiller et al. (2007) also found a similar targeting frequency for another moss, suggesting that this might be a conserved feature of bryophyte transformation. Finally, the completion of the genome sequence (Rensing et al., 2008) reinforced the model plant status of *P. patens*. However, the genome sequencing and reverse genetics have revealed a large number of recently duplicated genes exerting redundant functions and thus, some developmental biologists now look for alternative plant models among the bryophytes, e.g. liverworts of the genus *Marchantiales*.

1.9.1 Life cycle of *Physcomitrella patens*

The life cycle of *P. patens* has been described in detail by Reski (1998) and is here summarized if Figure 5. Like all embryophytes *P. patens* alternate

between a haploid gametophyte, that produces gametes, and a diploid sporophyte, producing haploid spores. Germination of the haploid spore generates the haploid gametophyte generation. The gametophyte consists of a filamentous tissue called protonemata and more angiosperm-like structures called gametophores, or leafy shoots. The protonemal filaments grow by apical cell divisions and sub-apical branching, and have generally a very simple organization, consisting of only two cell types, chloronema and caulonema. The first cell type to be produced by the spore is the chloronemata, with many chloroplasts, thus having assimilatory functions. From the chloronemal filaments caulonemal filaments are produced by gradual transition of the apical cell. The caulonemal cells are longer and grows faster than chloronemal cells, however they do not contain as many chloroplasts and hence the function is not in energy assimilation, instead these filaments grow out from the colony centre to occupy new territory.

The sub-apical cells of protonemata can develop side-branch initials with the potential to produce new organs. Unlike chloronemal side branch initials, which can only develop into new chloronemal filaments, caulonemal side-branch initials also produce chloronemal filaments and buds, in addition to new caulonemal filaments. The bud differentiates and gives rise to the gametophore. The gametophore is an upright structure consisting of a stem, leaf-like single cell-layer structures referred to as leaves, and rhizoids (caulonema-like filaments) attached to the base and lower part of the stem. The gametophore does not have a true vasculature, although there are conductive cells in the stem. The rhizoids have been suggested to mainly have structural functions, in keeping the gamatophore attached to the ground, but other functions cannot be excluded. A recent study showed that homologous developmental programs regulate formation of root-hairs in *A. thaliana* and rhizoids in *P. patens*, suggesting that rhizoids are homologous to root-hairs (Menand et al., 2007). The gametophore of *P. patens* also has small stipule-like organs referred to as axillary hairs. These single-celled structures are attached to the leaf-stem junction and can also be found in other mosses. However, the size and complexity of the axillary hairs is highly variable between different moss species (Schofield and Hebant, 1984). For *P. patens* and other mosses it has been suggested that the axillary hairs produce mucilage, which might protect the moss from drying out, however this remains to be proven experimentally (Schofield and Hebant, 1984).

Mosses are, just as other plants, either monoceious or diceious and because *P. patens* is monecious both type of reproductive organs are formed in the stem apex of the same mature gametophore during low energy conditions, e.g. short days and low temperature. The male and female

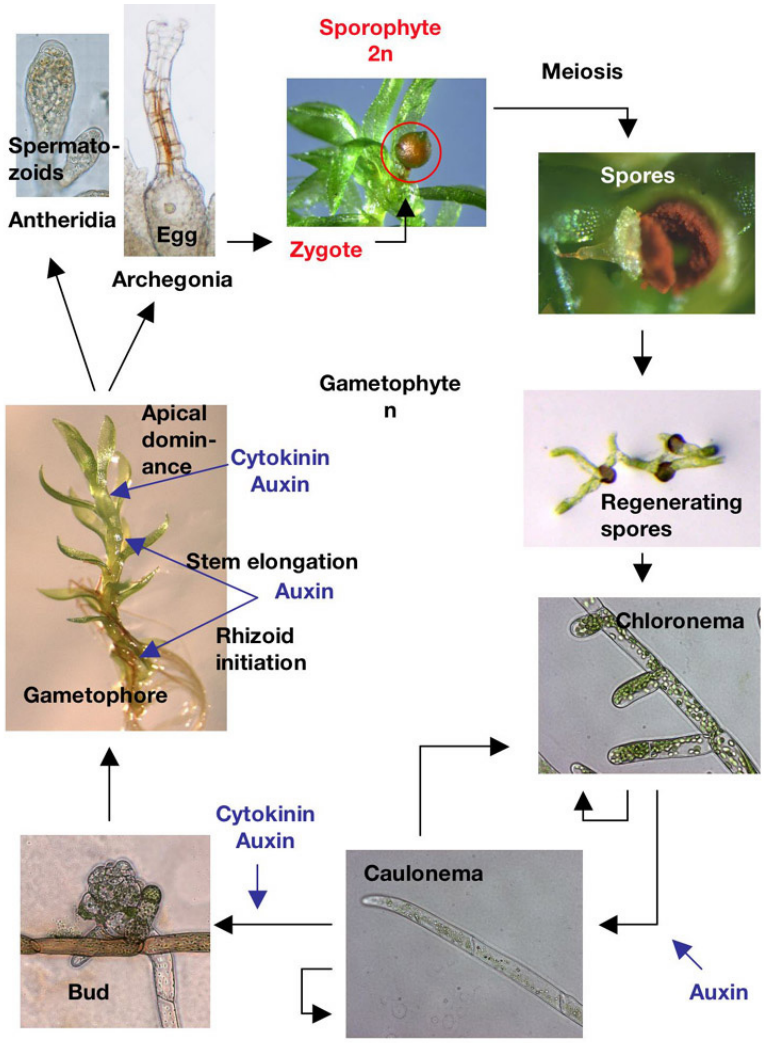

Figure 5. The life cycle of *P. patens* and its hormonal regulation. Photos by Anders Nilsson and Mattias Thelander.

reproductive organs, antheridia and archegonia, produce spermatozoids and eggs, respectively. The diploid zygote is formed after fertilization, a process that requires water because the spermatozoids are equipped with flagella to facilitate its movement from the male (antheridia) to the female (archegonia) reproductive organs. The zygote develops into an embryo and gives rise to a short stem (seta), carrying the spore capsule (sporangium). The cells in the spore capsule undergo meiosis to produce some 4000 haploid spores. The diploid sporophyte generation is thus restricted to a small and short-lived

spore-producing structure that is dependent on the gametophyte generation for physical support and nutrients (Cove, 2005).

1.9.2 Hormonal regulation of moss development

Although the bryophytes diverged from seed plants at the very beginning of embryophyte evolution, growth and development in moss appear, at large, to be regulated in a similar fashion as in angiosperms, by factors such as gravity, light, temperature, nutrient supply, stress and hormones (Imaizumi et al., 2002; Ashton et al., 1979; von Schwartzenberg et al., 1998; Knight et al., 1995; Nagao et al., 2005; Wang et al., 2008). Analysis of the *P. patens* genome sequence does however indicate major differences between moss and angiosperms in some key developmental mechanisms, i.e. *P. patens* appear to lack the ability to produce, and respond to, several hormones found in angiosperms.

Homologues to genes from vascular plants encoding rate-limiting enzymes for GA synthesis are found in the *P. patens* genome (Hayashi et al., 2006; Rensing et al., 2008), however, the GA receptors (i.e. GID-like proteins) in *P. patens* cannot bind GA, in contrast to the GA-binding GID-like GA receptors of *S. moellendorffii*, and no distinct effects of GA on moss growth has been reported (Hirano et al., 2007). Hence, GA signaling in moss is probably not similar to that of higher plants. Also, genes likely to encode key enzymes involved in JA, BR, IAA, ET, ABA and CK synthesis are thought to exist (Rensing et al., 2008), but distinct effects of exogenous hormone application on moss growth and development has only been reported for auxin, CK and ABA (reviewed in Cove et al., 2006; Decker et al., 2006). Although no definite role for ABA in moss development has been established it has been reported that high exogenous levels of ABA inhibit bud formation, in addition to its well-characterized effects related to responses to abiotic stress (Cristianson, 2000; Decker et al., 2006; Cove et al., 2006). Interestingly, although there are putative ET receptors and ACC synthetases (rate-limiting for ET synthesis in higher plants) in moss, no effects of ET or ACC (1-Aminocyclopropane-1-Carboxylate, an ET precursor) treatments are known.

Knowledge of the biological functions of hormones in moss is thus still rudimentary, however, roles for CK and auxin has been extensively studied and will be described next.

Regulation of moss development by cytokinins

CK is known to induce bud formation in more than 20 different moss species, including *P. patens* (Figure 5; Gorton and Eakin, 1957; Bopp and

Brandes 1964; Cove and Ashton, 1984; Ashton et al., 1979b). Consequently lack of CK results in continuation of filamentous growth by the creation of new caulonemal and chloronemal filaments from the caulonemal side-branch initials. As previously mentioned, buds are usually formed on caulonemal filaments, but exogenous CK treatment also induce buds on chloronemal filaments in a concentration dependent manner (Ashton et al., 1979b; Reski and Abel, 1985) and high concentrations of CK induce the formation of numerous callus-like buds that never develop into adult gametophores (Reski, 1998). Additionally, several mutants showing gametophore overproduction have been described (Ashton et al., 1979a) and one class of these mutants was shown to over-produce zeatin and isopentenyadenin (both of which are CK; Wang et al., 1980, 1981). These mutants can affect neighboring colonies to induce overproduction of buds (Wang et al., 1980, 1981) which fits with the finding that the majority of the CK produced by protonemal filaments of the wild type under normal growth conditions is transported out of the cells into the growth substrate (Reutter et al., 1998). CK has also been reported to repress outgrowth of adventitious gametophores on the gametophore stem (Nyman and Cutter, 1981).

In conclusion, the only established biological functions of CK in moss development is thus to maintain apical dominance and to induce the transition from filamentous growth to leafy shoots carrying the capacity of sexual reproduction.

Regulation of moss development by auxin

The transition from chloronema to caulonema is induced by exogenous auxin (Johri and Desai, 1973) and is reverted by exogenous anti-auxin (PCIB; Sood and Hackenberg, 1979). Additionally, Ashton et al. (1979b) produced several non-allelic auxin resistant mutants unable to form caulonema or buds, verifying the biological function of auxins in the chloronema to caulonema transition (Figure 5). This transition is also induced by high-energy conditions i.e. high light intensity and excess sugars (Thelander et al., 2005). However, also the latter effect may well be mediated by auxin because exogenous application of auxin in high light conditions have little effect (Rose et al., 1983), suggesting that auxin signaling is already active and saturated in high light.

Interestingly, approximately 90% of the auxin produced by moss protonemal filaments could be found extracellularly, just as for CKs (Reutter et al., 1998). The physiological relevance of the secreted hormones was demonstrated by continuous media exchange resulting in abolished auxin-

mediated differentiation of protonemal filaments (Ashton et al., 1979b; Schween et al., 2003). It thus appears that normal protonemal development is dependent on extracellular auxin, and CK, located on the plant or in the growth substrate. As expected, differentiation to caulonema is reinitiated by the addition of exogenous auxin and CK (Cove and Ashton, 1984).

Auxin has also been shown to be essential for bud formation together with CK (Figure 5). No buds are formed in CK-resistant mutants, after exogenous CK application (Ashton and Cove, 1990; Ashton et al., 1979b). However, exogenous auxin can restore bud formation in these mutants, suggesting that they might be auxin biosynthesis mutants and that in addition to CK, bud assembly requires auxin (Ashton and Cove, 1990; Schumaker and Dietrich, 1997).

As previously mentioned auxin-mediated processes of angiosperms include organ initiation, apical dominance and axis elongation. All these features of auxin action can also be found in gametophores of moss (Nyman and Cutter 1981; Bopp 1983). Hence, in addition to inducing chloronema to caulonema transition and bud formation, exogenous auxin also affect gametophore development by inducing formation of rhizoids (Sakakibara et al., 2003), promoting stem elongation (Ashton et al., 1979b) and, together with CK repress the formation of adventitious gametophores on the gametophore stem (Figure 5; Nyman and Cutter, 1981). Also decapitation of gametophores, removing the apical part of the stem, induces formation of adventitious gametophores and this process is repressed by exogenous auxin together with CK, but not by auxin alone (Nyman and Cutter, 1981).

Evidence of auxin biosynthesis in P. patens

There are 71 *CYP450* genes in *P. patens*, compared to the 246 genes in the genome of *A. thaliana*, however, none of the moss genes appear to be a close homologue to any of the four *CYP450* genes belonging to the *CYP79* subgroup in *A. thaliana* (Nelson, 2006). Additionally, to my knowledge no *TAA1*-, *TDC*-, *AMI1*- or *NIT*-like genes, or any other genes described or proposed to act in IAA biosynthesis in *A. thaliana* have yet been reported in moss. The only exception being the six *YUC*-like genes described previously by the moss-sequencing consortium (Rensing et al., 2008) and by Gallavotti and co-workers (2008).

In an attempt to establish which Trp-dependent IAA biosynthesis pathways that exist in moss, Lehnert and Bopp (1983) fed moss protonemal tissue with different IAA precursors. This study used the fact that auxin, in combination with CK, is required for bud formation in moss, and effect of the different precursors was measured by their ability to induce buds on

caulonemal filaments. This study clearly showed that exogenous IAN or TAM, in combination with CK, were unable to stimulate bud formation in low light, while buds were formed after application of Trp, IAAld, IPA and IAA (Lehnert and Bopp, 1983). This suggests that auxin producing pathways using TAM and IAN as substrates are not active in protonemal filaments. Additionally, in the study by Lehnert (1982), using similar methods as previously described, the author suggests that the IAM pathway is present in moss. These reports provide some information concerning IAA synthesis in moss protonemata, however, various factors such as uptake and stability could explain differences in bud forming activity between the different precursors.

Taken together, very little is known of the different auxin biosynthesis pathways operating in moss.

Auxin homeostasis in moss

In higher plants more than 90% of all IAA can be found in various conjugated forms, but in mosses the average relative value of several species is only 70% (Sztein et al., 1999, 2000). Mosses thus conjugate auxin, however the mechanism appears to operate slower than in higher plants, suggesting that conjugates may play a different role in auxin homeostasis in mosses compared to higher plants (Sztein et al., 2000; Cooke et al., 2002). Additionally, charophytes (a group of algae), hornworts and liverworts conjugate auxin even slower, indicating that the impact of conjugation on auxin homeostasis becomes decreasingly important the farther away from vascular plants you get (Cooke et al., 2002). Additionally, the impact of biosynthesis on homeostasis appears to have a negative correlation to the impact of conjugation (Cooke et al., 2002).

P. patens contain two *GH3*-like genes which both are expressed in the gametophyte. They have been reported to cluster with the *A. thaliana GH3-11/JAR1* gene, encoding an enzyme shown to conjugate JA (Imaizumi et al., 2002; Bierfreund et al., 2004; Staswick and Tiryaki, 2004; Staswick et al., 2005). Only one of the *GH3* genes in *P. patens* can convert IAA to amide conjugates efficiently *in vitro,* whereas both enzymes can use IBA or JA as substrates (Ludwig-Müller et al., 2009). Interestingly, IBA has not been reported to exist in moss (Ludwig-Müller, 2000) and the biological function of JA is unknown.

Knock-out mutants of the two *GH3* genes in *P. patens* have been analyzed in respect to auxin levels and auxin sensitivity (Bierfreund et al., 2004; Ludwig-Müller et al., 2009). The *GH3* single knock-out mutants do not have any obvious phenotypes (Bierfreund et al., 2004) but the growth

inhibiting effect of exogenous auxin is more pronounced in the single mutants than in wild type, indicating that the mutants have problems inactivating excess auxin. The mutants also show similar defects in IAA conjugation suggesting overlapping functions *in planta* (Ludwig-Müller et al., 2009). The double mutant, having no GH3 activity, shows amide conjugate levels close to zero. However, the double mutants still show conjugation activity and the levels of ester conjugates are high. This, and the presence of only two *GH3*-like genes in moss compared to the 19 in *A. thaliana,* suggests mechanisms other than amide conjugation to be more important for the control of auxin homeostasis in moss.

Ludwig-Müller et al. (2009) also showed that the GH3 protein probably only have a function for IAA homeostasis in the gametophore and not in protonemal filaments, suggesting that GH3-mediated amide conjugation only have effects on auxin homeostasis during specific stages of moss development.

Apart from the *GH3*-like genes and their function in creating amide conjugates to reduce free IAA levels not much is known. There are however UGT homologues in *P. patens* that could have a function in the creation of ester conjugates (Ludwig-Müller et al., 2009), but nothing is known of hydrolysis of conjugates or the terminal inactivation/degradation/catabolism of IAA and IAA conjugates. Finally, it would be interesting to know if auxin secretion/uptake has a role in homeostasis, as a mechanism to reduce/increase intracellular auxin levels.

Auxin transport and signaling in moss

Several homologues of genes regulating auxin action in *A. thaliana* exist also in *P. patens,* e.g. genes encoding homologues of the receptors TIR/AFBs and ABP1, the transport facilitators AUX1/LAXs and PINs, and the transcriptional regulators AUX/IAAs and ARFs (Rensing et al., 2008). These findings suggest that the mechanisms underlying auxin transport, perception and signaling is similar in mosses and higher plants. Also the number of genes in each of the mentioned gene families is not dramatically different compared to *A. thaliana* (Rensing et al., 2008; Floyd and Bowman, 2008), with the exceptions being the AUX/IAA transcriptional repressors and AUX1/LAX auxin influx facilitators of which 2 and 8 genes can be found in *P. patens,* respectively, compared to the 29 and 4 genes found in *A. thaliana*. Additionally, so called, anti-auxins targeting the function of TIR1/AFBs affect *P. patens* in similar ways as *A. thaliana* (Hayashi et al., 2008), verifying similar mechanisms of auxin perception.

A basipetal auxin gradient has been suggested to form in protonemal filaments and to play a role in caulonema differentiation (Schwuchow et al., 2001). However, this is only based on import/export of auxin in protonemata and the apparent existence of export- and import facilitators, and thus this gradient remains very speculative (Bopp and Atzorn, 1992; Rose et al., 1993; Rose and Bopp, 1983). The export/import does not necessarily implicate cell-to-cell PAT, but could be a way to transport auxin in and out of the protonemal cells to the surrounding media. The existence of auxin fluxes has also been identified in protoplasts where NPA or TIBA treatments reduced auxin efflux and increased cellular auxin levels (Bopp and Geier, 1988; Geier et al., 1990). NPA has also been shown to strongly inhibit gravitropic curvature of the apical cell of moss protonema (Schwuchow et al., 2001). Because NPA has not been shown to affect unicellular curvature in higher plants the PIN auxin efflux machinery might operate differently in mosses compared to *A. thaliana* (Schwuchow et al., 2001). Additionally, basipetal PAT has been suggested to take place in rhizoids creating an auxin gradient with maxima in the basal cells (Rose and Bopp, 1983). PAT has also been demonstrated in the sprorophyte (Fujita et al., 2008). Interestingly, Fujita and co-workers (2008) suggested that no long range PAT occurs in the stem of the leafy shoots in any of the moss species tested. They could show that application of NPA did not have any effect on leafy shoot development and that radio-labeled auxin applied to the ends of stem segments did not migrate longer in the stem than what is expected if only transported by diffusion. Since no long range PAT has been detected in the leafy shoot it would be very interesting to know how auxin is transported and how presumed auxin gradients are created and maintained in this structure.

To create a tool for monitoring changes in auxin signaling in moss, Bierfreund et al. (2003) produced lines carrying auxin responsive promoter-GUS fusions. Auxin responsive constructs, e.g. *GH3pro:GUS*, are widely used in *A. thaliana* to monitor the auxin signaling capacity of a specific tissue (Hagen and Guifoyle, 1985; Hagen et al., 1991). In *P. patens* protonemal filaments and gametophores it has been shown that both *DR5pro:GUS* and *GmGH3pro:GUS* constructs can respond to exogenous auxin by an increased and ectopic GUS signal (Bierfreund et al., 2003). The *GmGH3* promoter, however, show higher response capacity compared to *DR5* in moss, and is thus suggested to be the better option for studies of auxin signaling. It is also shown that the auxin signaling hot-spots in moss protonemata during normal growth is in the caulonemata and for the gametophore it is in the stem apex, the axillary-hairs, the base of the

gametophore and in the basal parts of the rhizoids, as judged by *GmGH3* promoter activity (Bierfreund et al., 2003; Fujita et al., 2008). Fujita et al. (2008) also showed that wounding quickly induced auxin signaling in the gametophore stem.

2 Aims of the present study

The aim of my work has been to investigate auxin-mediated regulation of plant growth and development and more specifically to gain insight into how regulators of auxin biosynthesis operate. This was achieved by functional characterization of members of the *SHI/STY* family in general, and *STY1* in particular.

The more specific aims were to:

- Investigate the role of SHI/STY family members in regulation of auxin biosynthesis by:
    - Identifying and characterizing interactions between SHI/STY members and downstream target genes.
    - Identifying and characterizing factors regulating expression of *SHI/STY* family genes.
- Identify, characterize and compare *SHI/STY*-like genes in *A. thaliana* and *P. patens* to gain further understanding of the fundamental, and ancestral, mechanism regulating auxin-mediated plant growth.

3 Results and discussion

3.1 The *SHI/STY* gene-family is limited to embryophytes (I, IV)

The ten members comprising the *SHI/STY* gene family in *A. thaliana* have previously been described (Fridborg et al., 1999; Fridborg et al., 2001; Kuusk et al., 2002; Smith and Fedoroff, 1995). The genes are referred to as *SHORT INTERNODES* (*SHI*), *STYLISH1* and *2* (*STY1* and *STY2*), *LATERAL ROOT PRIMORDIUM1* (*LRP1*) and *SHI-RELATED SEQUENCE3* to *8* (*SRS3* to *SRS8*). The proteins they encode all have very similar organization and share two conserved domains, a putative RING-like zinc-finger (ZnFn) and an IGGH domain of unknown function (Fridborg et al. 2001). All SHI/STY proteins also share a nuclear localization signal (NLS) and various glutamine-rich (Q-rich) regions.

We have previously observed that this gene family is plant specific as no homologues have been found in other kingdoms than the plant kingdom, *Viridiplantae* (Fridborg et al., 2001). When searching the available databases we found *SHI/STY* members in all analyzed multi-cellular land plants, among them two genes in the bryophyte *Physcomitrella patens,* and three in the lycophyte *Selaginella moellendorffii*. The moss genes were named *PpSHI1* and *PpSHI2*. Interestingly, we failed to identify any *SHI/STY*-related genes in algae of the chlorophyte group, indicating that this gene family is restricted to the sub-group of embryophytes in the plant kingdom. We have however not analyzed members of the charophyte group.

Furthermore, the number of *SHI/STY*-related genes in embryophytes appears to correlate with the complexity of the body plan of the species. *P. patens*, being simple in structure, and non vascular, having two *SHI/STY* genes, *S. moellendorffii*, with a primitive vasculature, having three, and

vascular plants such as *A. thaliana*, rice (*Oryza sativa*) and Poplar (*Populus trichocarpa*) having nine, five and nine *SHI/STY*-like genes, respectively (Figure 6; One of the *A. thaliana* members is a pseudogene (section 3.2) hence 9, not 10, in the figure).

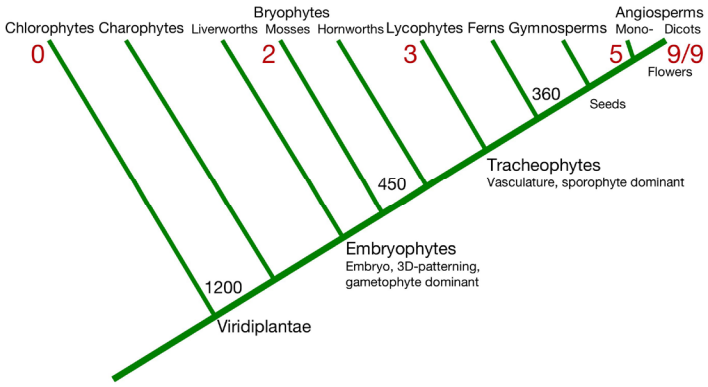

Figure 6. The number of *SHI/STY* family genes (red numbers) correlate with the complexity of the plant in the examined species. Very simplified view of the kingdom Viridiplantae to show the relation and evolutionary distance between *P. patens* (bryophyte), *S. moellendorffii* (lycophyte), rice (moncot) and *A. thaliana* and poplar (dicots). Black numbers indicate million years since the groups were separated.

3.2 Phylogeny of the *SHI/STY*-family (I, IV)

Transcripts of *SHI, STY1*, *STY2* and *LRP1* have previously been isolated (Fridborg et al., 1999; Kuusk et al., 2002; Smith and Fedoroff, 1995). To confirm that the remaining *SHI/STY* genes in *A. thaliana* are active during plant development we analyzed transcripts of *SRS3*-7 by RT-PCR. All these genes are active in seedlings, leaves, flowers and siliques, except for *SRS3,* which could not be detected in leaves, and *SRS5,* which could not be detected in siliques.

SRS8 is located in the centromeric region of chromosome 5, surrounded by pseudogenes. We were never able to PCR-amplify an *SRS8* transcript and thus, we consider *SRS8* a pseudogene.

We analyzed the phylogeny of the nine expressed *SHI/STY* genes in *A. thaliana*, as well as *SHI/STY* members from other species, such as rice and poplar, using different methods. The resulting trees all showed similar topology with three distinct pairs: *SHI/STY1*, *STY2/SRS4* and *SRS5/SRS7,* among the *A. thaliana* members. These pairs were also supported by their chromosomal locations on duplicated genomic regions,

indicating an origin of several *SHI/STY* members in the most recent genome duplication event.

The *SHI/STY* family appears split into two major clades, supported by high bootstrap values. One clade harbors *SRS6* and *LRP1*, while the other seven *A. thaliana* members are found in the other clade. The two *P. patens* and three *S. moellendorffii* orthologs group with the *LRP1* and *SRS6* clade, suggesting this clade to be the ancestral one. This also suggests that the vascular plant-specific clade might be a result of sub-functionalization and that functional differences between moss and, at least some, *A. thaliana* *SHI/STY* members might exist.

Additionally, the *P. patens* genes group together, likely as a result of a recent genome duplication event in the moss lineage (Rensig et al., 2007). A similar clustering is observed for the three *S. moellendorffii* genes, suggesting that the common embryophyte ancestor had one *SHI/STY* gene.

3.3 *SHI/STY*-family expression patterns in *A. thaliana* (I)

The spatial and temporal expression patterns of *SHI*, *STY1*, *STY2* and *LRP1* have previously been extensively investigated using reporter constructs, *in situ* hybridization and RT-PCR (Fridborg et al., 2001; Kuusk et al., 2002; Smith and Fedoroff, 1995).

LRP1 has previously been described as being expressed only in lateral root primordia (LRP; Smith and Fedoroff, 1995), however, we detected *LRP1* transcript in flowers using RT-PCR. This was supported by microarray data indicating *LRP1* expression in apices, roots, carpels and other parts of the flowers (Schmid et al., 2005). Thus, the expression reported for the GUS-transposon tagged *LRP1* gene (Smith and Fedoroff, 1995) does not reflect the endogenous *LRP1* expression pattern. Interestingly, in the LRP1-GUS line (*lrp1*) the *uidA* gene is inserted just before the transcriptional start, followed by some 4kb of transposon sequence. Thereby, the regulatory role of putative *cis*-regulatory elements in e.g. the 5'UTR is not accounted for in *lrp1* (See section 3.7 for further details regarding putative roles of the *LRP1* 5'UTR in transcriptional regulation).

The expression patterns of SHI_{pro}*:GUS*, $STY1_{pro}$*:GUS* and $STY2_{pro}$*:GUS* have been reported as being highly overlapping (Fridborg et al., 2001; Kuusk et al., 2002). To analyze the expression pattern of a member of the third pair, we made a $SRS5_{pro}$*:GUS* construct and could observe that its expression overlaps with the other GUS fusions, suggesting a high level of functional redundancy. All four GUS fusions showed expression in LRPs,

young rosette leaves, hydathodes and flowers, with some spatial and temporal deviations. In the gynoecium, *STY1* is expressed from stage 6, followed by *SHI* at stage 8 (Smyth et al., 1990; Fridborg et al., 2001; Kuusk et al., 2002). *STY2* and *SRS5* are expressed from stage 9 and 10, respectively (Kuusk et al., 2002). These, and other, dissimilarities in expression indicate differences in their impact on developmental processes and could be the result of a process of sub-functionalization.

The expression patterns of *SRS3*, *SRS4*, *SRS6* and *SRS7* are largely unknown. However, microarray data reveal that *SRS4* and *SRS6*, at least partially, overlap with the other *SHI/STY* genes (Schmid et al., 2005). Unfortunately, no satisfactory GUS fusions of *LRP1* or *SRS6* exist that could clarify potential differences between members of the two major clades.

3.4 Functional redundancy within the *SHI/STY* family (I, II, IV)

The *sty1-1* and *sty2-1* single mutants have previously been phenotypically characterized (Kuusk et al., 2002) and *shi-3* has previously been reported as being identical to wild type (Fridborg et al., 2001). *sty1-1* and *sty2-1* are to this day the only *SHI/STY* single mutants in *A. thaliana* in where we have been able to observe developmental defects. In *sty2-1* the leaves are slightly more serrated than what is typically seen in wild type. The phenotype for *sty1-1* can be described as a slight depression in the medial plane of the style and stigma, appearing during gynoecium development and remaining in the mature fruit. The other single mutant lines (*shi-3*, *srs3-1*, *srs4-1*, *srs4-2*, *srs5-1*, *srs7-1* and *lrp1*) appear to be knock-outs or knock-downs but are identical to wild type. However, deviations from wild type potentially could exist in these lines under conditions not yet analyzed. No mutant in *SRS6* has been characterized.

3.4.1 Gynoecium development

The phenotype of *sty1-1* could be linked to the unique temporal expression pattern of *STY1* in the style of the developing gynoecium and this would nicely explain why no other *SHI/STY* members can compensate for loss of *STY1* during gynoecium development. The depression in the stylar region of *sty1-1* was accompanied by a reduced vasculature in the style and a shift of vein bifurcation toward the base of the gynoecium (Kuusk et al., 2002). These effects were enhanced in the *sty1-1 sty2-1* double mutant indicating some degree of functional redundancy (Kuusk et al., 2002). However, *SHI* is more closely related to *STY1* than *STY2* is, and the *sty1-1 shi-3* double mutant showed more severe effects on stylar development than the *sty1-1*

sty2-1 mutant. This could also be an effect of temporal expression differences between *STY2* and *SHI*. For *sty1-1 shi-3* the stylar and stigmatic tissues were reduces and the apical region of the gynoecium was open.

We examined the ability of other *SHI/STY* family members to affect gynoecium development and observed that *srs4-1/2*, *srs5-1* and *lrp1* all enhance the gynoecium phenotype in *sty1-1* background, with varying effects on style development: The *sty1-1 lrp1* mutant displayed a distinct enhancement of the *sty1-1* phenotype while *sty1-1* in combination with *srs4-1*, *srs4-2* or *srs5-1* only showed a slight enhancement.

We also produced a number of triple and quadruple lines as well as a single quintuple mutant line. Interestingly, all multiple mutant combinations without *sty1-1* had gynoecia identical to wild type. When *sty1-1* was included a gradual enhancement was observed as more *SHI/STY* mutants were introduced. The most severely affected higher order *SHI/STY* mutant was the quintuple mutant (*sty1-1 sty2-1 shi-3 lrp1 srs5-1*) having e.g. completely unfused carpels, a stylar region with horn-like protrusions and a very reduced seed production. Additionally, several higher order mutants had an elongated gynophore. This, and the reduced seed set show that mutations in *SHI/STY* genes affect not only the apical part of the gynoecium but also the ovaries and the basal part of the gynoecium.

3.4.2 Leaf development

The leaves of *sty2-1* were more serrated than wild type, and this serration was enhanced as more *SHI/STY* mutations were introduced into the *sty2-1* background. In quadruple and quintuple lines radialized organs, resembling leaves, could sometimes be found. Leaf serration was dependent on *sty2-1*, as higher order mutants without *sty2-1* were identical to wild type.

3.4.3 Root development

Even though many *SHI/STY* genes are expressed very early during the initiation of LR formation, and in root tips, we have not been able to establish a function for *SHI/STY* genes in the root. We observed no variations from wild type roots in the *sty1-1 sty2-1 shi-3 lrp1 srs5-1* quintuple mutant, or in $35S_{pro}$*:STY1-SRDX* lines. However, because there are no flower or leaf phenotypes unless *sty1-1* respectively *sty2-1* are mutated, it is likely that we will find a root phenotype by introducing mutations in additional family members. It is also possible that simultaneous knock-down of several family-members, using RNAi, miRNA or a similar method, would result in developmental abnormalities in the root.

3.4.4 Regulation of floral organ identity

In *SHI/STY* family triple, quadruple and quintuple mutant lines we frequently observed developmental defects of floral whorls two (petals) and three (stamens). The petals were more sepal-like and the stamens were more carpel-like. The phenotypes resemble those of lines carrying mutations in the floral homeotic genes *PISTILATA* (*PI*) and *APETALA3* (*AP3;* Bowman et al., 1991). We analyzed the levels of *PI* and *AP3* transcripts in the quintuple mutant and found a significant decrease in the levels of both transcripts, confirming the phenotypic defects. Because AP3 and PI regulate each other's expression (Goto and Meyerowitz, 1994) our data indicate that *SHI/STY* members regulate one or both of these genes.

3.4.5 A STY1-SRDX fusion protein can override the action of redundant genes

To further analyze the role of *SHI/STY* genes in *A. thaliana* we created a STY1-SRDX fusion and expressed it ectopically in Columbia. SRDX is a small (12 amino acids) synthetic repressor domain first described by Hiratsu et al. (2003). SRDX has been fused to several transcriptional activators, e.g. *CUP-SHAPED COTYLEDON1*, *AtMYB23* and *PRODUCTION-OF-ANTHOCYANIN-PIGMENT1,* to convert them to repressors in order to overcome the problem of functional redundancy. These genes take part in different developmental processes and their respective SRDX fusions resulted in proteins acting in a dominant negative manner and the transformants therefore resembled multiple knock-down mutant lines (Hiratsu et al., 2003; Matsui et al., 2005). The dominant negative effect of repressor-transcription factor fusions has also been demonstrated using the *engrailed* repressor from *Drosophila* fused to genes such as *PISTILLATA*, *SHOOTMERISTEMLESS* and *APETALA3* (Markel et al., 2002). We hypothesized that if STY1 acts as a transcriptional activator, redundant in function with other SHI/STY members, a STY1-SRDX fusion would function as a dominant negative mutation and result in higher order *SHI/STY* mutant-like phenotypes. As expected we observed *SHI/STY* mutant-like phenotypes in cotyledons, gynoecia, petals, sepals and leaves.

3.4.6 Functional redundancy is a characteristic feature for the *SHI/STY* family

As further discussed below, *PpSHI1* and *PpSHI2* loss-of-function mutants displayed identical phenotypes and the expression domains were also identical for the two genes, suggesting that they exert similar, or maybe even identical, functions in moss development. We believe that SHI/STY

proteins influence developmental processes in a dose-dependent manner in *A. thaliana*. This is likely also the best explanation for the *SHI/STY* loss-of-function phenotypes observed in *P. patens*.

In conclusion, our data suggest the *SHI/STY* family in *A. thaliana* and *P. patens* to be highly redundant, although *STY1* and *STY2* have unique functions in development of gynoecia and leaves, respectively. Alternatively, the phenotypic effects of *sty1-1* and *sty2-1* could be caused by formation of a dominant negative truncated protein.

3.5 SHI/STY members are transcription factors (II, IV)

All *SHI/STY* family members putatively encode similar proteins, having a number of glutamine-rich regions, one zinc finger domain with two closely spaced zinc fingers resembling a RING-finger, an NLS and an IGGH domain containing an acidic region. The proteins are of intermediate size (35-40kDa) with a pI close to seven.

All these features suggest SHI/STY family proteins to act as DNA-binding transcriptional activators.

3.5.1 SHI/STY proteins are active in the nucleus

We could show that STY1 and *Pp*SHI1/2 were localized to the nucleus, using GFP and GUS fusions, suggesting that all SHI/STY members are nuclear proteins. In addition, Sohlberg et al. (2006) observed that ectopically expressed STY1 fused to the rat glucocorticoid receptor domain (GR) rescued the *sty1-1* phenotype in a GR-ligand (dexamethasone; DEX) dependent manner, giving indirect evidence that STY1-GR exert its function, or functions, in the nucleus.

3.5.2 SHI/STY proteins are transcriptional activators

Sohlberg et al. (2006) used the *$35S_{pro}$:STY1-GR* line to study global changes of the transcriptome after DEX treatment. It was observed that many genes were up regulated already after one hour, indicating a very fast transcriptional activation by DEX-mediated transport of STY1 to the nucleus (Sohlberg et al., 2006). We also used a *$35S_{pro}$:SHI-GR* line in a microarray analysis with similar results as for *$35S_{pro}$:STY1-GR* (unpublished data). The quick response after DEX treatment was later verified by quantitative PCR (Q-PCR) and here we have shown that this is not dependent on *de novo* protein synthesis, suggesting a direct activation by STY1 of the targeted gene (*YUCCA4*; See section 3.6). To verify the positive effect of SHI/STY members on transcriptional activation *in vivo*,

and to dissect the origin of the activation activity, we assayed various truncated STY1-GAL4BD fusion proteins for transactivation activity in yeast. This showed that parts of STY1 harboring an acidic or glutamine-rich region all could activate transcription. We also mutated the acidic amino acids in the IGGH domain of *Pp*SHI1 and found that the mutation reduced its ability to activate transcription in yeast to almost zero.

Additionally, the fact that a STY1-SRDX fusion protein can overcome functional redundancy strongly indicate that the *in planta* function of STY1 is to activate transcription.

3.5.3 SHI/STY members are DNA binding proteins

We used chromatin immunoprecipitation (ChIP) to show that DNA from the *YUC4* promoter co-precipitate with the STY1-GR fusion protein. The interaction between STY1 and the *YUC4* promoter was also observed using yeast one-hybrid assays. The ability of STY1 to interact with presumably identical promoter regions in two very different eukaryotes suggest STY1 to either directly, or indirectly, bind DNA.

In a yeast one-hybrid screen for proteins interacting with the jasmonate (JA) responsive region of the AP2/ERF transcription factor gene *OCTADECANOID-RESPONSIVE CATHARANTUS ROSEUS AP2 3* (*ORCA3)* in *Catharantus roseus* (trivial name Madagascar periwinkle) a putative SHI/STY orthologue was identified (vom Endt et al., 2007). This SHI/STY orthologue was found to directly interact with the *ORCA3* promoter region *in vitro*, however, not to the JA-responsive element, but to another uncharacterized region. The yeast one-hybrid and electro mobility shift assay (EMSA) preformed by vom Endt et al., (2007), together with our ChIP and yeast one-hybrid assays, indicate that proteins of the SHI/STY family are capable of physically binding DNA.

The baits in the two mentioned yeast one-hybrid assays only had one element in common: ACTCTAC. We mutated parts of this element in our bait fragment and found that STY1 was incapable of interacting with the mutated element, suggesting that STY1 and the *C. roseus* SHI/STY orthologue binds, at least parts of, this element in their respective baits.

Variations in the zinc finger of different SHI/STY members may indicate differences in DNA recognition. However, because constitutive expression of STY1-SRDX results in multiple *SHI/STY* family loss of function mutant phenotypes we can conclude that STY1 most likely bind the same promoters as other SHI/STY members.

3.6 SHI/STY family members are positive regulator of auxin biosynthesis in *A. thaliana* (II)

As mentioned previously several lines of evidence indicate SHI/STY transcriptional activators to affect expression of the *YUC4* gene encoding an enzyme thought to be rate-limiting in Trp-mediated auxin biosynthesis (Zhao et al., 2001). In addition to this, we can also show that higher order *SHI/STY* loss-of-function mutants and the STY1-SRDX lines cause severe auxin-related defects in leaves, flowers, cotyledons etc. Also, previous studies suggest *SHI/STY* genes to be of importance for auxin homeostasis. The *sty1-1* mutant is hypersensitive to reduced PAT and to altered auxin signaling (Sohlberg et al., 2006). *SHI/STY* mutants are rescued by microapplication of NAA to the stigma/style (Ståldal et al., 2008) and many phenotypes related to those of *SHI/STY* mutants have also been reported for *YUC* loss-of-function mutants (Chang et al., 2006, 2007). This connection between *SHI/STY* and *YUC* members is further strengthened by the strikingly overlapping expression domains observed in *A. thaliana* (Chang et al., 2006, 2007; Kuusk et al., 2002). It was also observed that levels of several IAA metabolites are reduced in *SHI/STY* family mutants and levels of free IAA, as well as IAA biosynthesis rates, are elevated after DEX-mediated induction of STY1-GR in *A. thaliana* (Sohlberg et al., 2006; Ståldal et al., 2008). Finally, transcript levels of *YUC4* are reduced by approximately 50% in floral buds of the *sty1-1 sty2-1* mutant (Sohlberg et al., 2006), a reduction that probably not can be explained by a reduction of the style tissue in the *sty1-1 sty2-1* double mutant compared to wild type.

In addition to the STY1-*YUC4* interaction, we also found that *YUC8* and the AP2/ERF family member *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59* (*ORA59)* had putative STY1 binding sites and were induced after DEX-mediated nuclear translocation of STY1-GR. Not much is published regarding *YUC8*, but *ORA59* has been described as a MeJA and ET induced transcription factor, regulating genes involved in pathogen responses (Pré et al., 2008). Several of the genes induced by ORA59 i.e. *IGPS*, *TSB1* and *TSB2*, regulate Trp synthesis (Figure 1). Thus, *ORA59* has been associated with production of indole-related secondary metabolites. However, the ORA59 mediated Trp may also be used for production of IAA suggesting that *ORA59* could be an important regulator of Trp-dependent IAA biosynthesis. Also, as mentioned above, the putative SHI/STY orthologue in *C. roseus* was shown to bind to the promoter of *ORCA3*, an AP2/ERF encoding gene (vom Endt et al., 2007). The ORCA3 protein was shown to up-regulate transcription of a TDC

encoding gene, mediating the formation of TAM from Trp, in *C. roseus* (van der Fits and Memelink, 2000; Figure 1).

Taken together our data gives evidence for a direct regulation of *YUC4*, and thus IAA levels, by STY1 and suggest that SHI/STY members in *A. thaliana* can activate IAA biosynthesis, possibly by affecting production of enzymes functional at several different nodes of the IAA biosynthesis pathway.

3.7 A STY1-SRDX fusion protein reveals novel roles for *SHI/STY* genes in plant development (II)

When analyzing the lines constitutively expressing *STY1-SRDX* we found a few abnormalities previously not described for the *SHI/STY* family quintuple mutant, but possibly related to auxin effects. Two of them will be described here.

The first, and most striking, phenotype was the lack of a functional SAM and seedling lethality. *STY1* and possibly other *SHI/STY* genes are expressed in the embryo from the globular stage and on, at the site where cotyledon primordia are formed (Kuusk et al., 2002), and because the loss of a SAM was observed in the majority of the T1s from the $35S_{pro}$*:STY1-SRDX* transformation, we speculate that this phenotype correlates with a reduction of SHI/STY family target genes in the cotyledon initiation sites and primordia. Also the narrow cotyledons of these individuals showed an apparent lack of polarity. Interestingly, the two HD zip Class III members *CORONA* and *PHAVOLUTA*, with roles in determining leaf polarity and SAM establishment/maintenance (Prigge et al., 2005), has the putative STY1 binding site in their upstream regulatory regions. These genes could thus be targeted by STY1-SRDX, resulting in reduced transcription levels. However, another explanation could be that the SAM defects are the result of STY1-SRDX expression in the SAM where it normally should not be (Kuusk et al., 2002). Future studies using more specific expression of *STY1-SRDX*, e.g. $STY1_{pro}$*>>STY1-SRDX* lines, will hopefully answer any questions related to the function of *SHI/STY* genes during embryo development.

Secondly, in several of the $35S_{pro}$*:STY1-SRDX* lines we found siliques having styles with striking resemblance of the styles of *tower of pisa/ngatha* loss-of-function mutants (Weigel et al., 2000; Alvarez et al., 2006; Cristina Ferrándiz, personal communication; Figure 7). The TOP/NGA family consists of four redundant B3–domain transcription factors and constitutive expression of *TOP* genes results in phenotypes similar to those of *SHI/STY*

genes. *TOP/NGA* single mutants display narrow and elongated styles, which, due to an unequal valve growth, are tilted. Occasionally, the single mutants show more severe abnormalities i.e. unfused valves and reduced styles, much like higher order *shi/sty* mutants. However, this is more frequently observed in *TOP/NGA* double mutants than in the single mutants (Cristina Ferrándiz, personal communication). Interestingly, one of the *TOP/NGA* genes has the putative STY1 binding site in the distal promoter region and this gene is also significantly up-regulated after induction of STY1-GR. The putative connection between *SHI/STY* and *TOP/NGA* members remains to be established but the data presented here suggest that STY1 may regulate transcription of at least one gene of this gene family.

Figure 7. top1-1-like siliques of the *35S$_{pro}$:STY1-SRDX* class IV lines.

3.8 Expression of *SHI/STY* members in *A. thaliana* is regulated via a GCC-box and by the transcription factor LEUNIG (I, III)

To further understand the role of *SHI/STY* genes in auxin regulated development of *A. thaliana* we searched for upstream regulators of *SHI/STY* genes.

We found a 14 or 15 nt GCC-box-like element, from now on referred to as the GCC-box, conserved in 8 of 9 members of *A. thaliana*. This GCC-box is essential for expression of *STY1* in all aerial organs and tissues where auxin is reported to occur, as demonstrated by mutated *STY1$_{pro}$:GUS* reporter lines. Thus, mutations in the GCC-box did not affect expression in proximal cotyledon regions, hypocotyls and in the root. These findings are

consistent with the fact that the *lrp1* line, expressing a GUS gene on a transposon located close to the transcriptional start site of the *LRP1* promoter, did not show GUS signal in aerial organs (Smith and Fedoroff, 1995), because the GCC-box in *LRP1* is located in the 5'UTR.

The GCC-box element found in most *SHI/STY* members was not found in any other coding or regulatory region in *A. thaliana*. However, it was also found in *SHI/STY* members of rice, poplar and grape but not in the lycophyte, and primitive tracheophyte, *S. moelendorffii,* or in the bryophyte *P. patens*. It thus appears as if this specific GCC-box is restricted to *SHI/STY* genes of vascular plants.

Because GCC-box variants are established regulatory elements in pathogen defense signaling, and has been shown to be the target of AP2/ERF proteins induced by ET (Ohme-Takagi and Shinshi, 1990; Riechmann and Meyerowitz, 1998; Sakuma at al., 2002), we analyzed the response of *STY1* to exogenous ACC. No significant induction was however observed, suggesting that the *SHI/STY* GCC-box most likely is not an ET inducible element, and that it thus might not be involved in the same kind of defense related responses as other similar GCC-box elements. *STY1* did however respond to exogenous IAA, suggesting that *STY1*, and perhaps other *SHI/STY* members, might be involved in a positive feedback loop, resulting in increased local IAA biosynthesis. However, putative auxin-ET cross-talk in the etiolated seedling complicates things and a more detailed analysis need to be performed.

The AP2/ERF transcription factor DRNL (Kirch et al., 2003) have been shown to induce *SHI* (Ikeda et al., 2006; March-Martinez et al., 2006), also in the presence of an inhibitor of protein translation (Ikeda et al., 2006), suggesting that DRNL proteins bind and activate the promoter of *SHI* and maybe other *SHI/STY* genes. We found that ectopic DRNL induction could activate ectopic *STY1* and *SHI* expression and that this induction was GCC-box dependent. We could also show that the seedling phenotype associated with ectopic DRNL activity was totally blocked in the *sty1-1 sty2-1 shi-3 lrp1 srs5-1* mutant background, verifying that this phenotype is *SHI/STY*-dependent. However, we were not able to observe any reduction in the levels of $STY1_{pro}$*:GUS* or SHI_{pro}*:GUS* expression in the *drn drnl* double mutant background, suggesting that other proteins, most likely of the AP2/ERF family, are redundant to DRN and DRNL.

Two other putative regulators of *SHI/STY* activity is the transcriptional co-repressor LEUNIG (LUG; Liu and Meyerowitz, 1995; Conner and Liu, 2000) and its interaction partner SEUSS (SEU; Franks et al., 2002; Sirdhar et al., 2004; Ståldal et al., 2008). These two have been shown to repress

target genes putatively by interacting with their promoters via a third, DNA-binding, protein. *lug* and *seu* mutants show similar gynoecium defects as *sty1-1 sty2-1*. Interestingly, gynoecia of the *sty1-1 sty2-1 lug* triple mutant was identical to that of *lug* indicating that *lug* is epistatic to *sty1-1 sty2-1* and that *SHI/STY* members may act in the same pathway as *LUG*. We found that expression of *LUG* was unaffected in the *sty1-1 sty2-1* double mutant and in the quintuple mutant, suggesting that *STY1* and *STY2* do not act as regulators of *LUG*. Additionally, expression of *STY1* was reduced in the lug mutant and the stylar defects in *lug* $35S_{pro}$*:STY1-GR* were restored after DEX-mediated induction of ectopic STY1-GR activity, suggesting that *LUG* act upstream of *STY1* and auxin biosynthesis.

3.9 The moss *Physcomitrella patens* as a model to study evolution of auxin-mediated developmental processes (IV)

To learn more about the evolution of auxin-regulated developmental mechanisms we decided to generate and study loss- and gain-of-function lines of *PpSHI1* and *PpSHI2*. We also analyzed *PpSHI1* and *PpSHI2* expression domains using GUS and GFP reporter-lines, and global auxin responses using a *GmGH3*$_{pro}$*:GUS* reporter line (Bierfreund et al., 2003). Finally, auxin metabolites were quantified. We hypothesized that these studies would lead to a better understanding of auxin function in the ancestral land plant.

3.9.1 Expression of *SHI/STY* orthologues in moss overlap with sites of high auxin signaling

We utilized the efficient native system of homologous recombination in *P. patens* to tag the endogenous *PpSHI1* and *PpSHI2* loci with GFP and GUS, respectively. This resulted in a *PpSHI1*$_{pro}$*:PpSHI1-GFP* line and several *PpSHI2*$_{pro}$*:PpSHI2-GUS* lines, all being morphologically identical to wild type. All observations of GUS and GFP signals in these lines suggest a complete overlap of *PpSHI1* and *PpSHI2* expression domains in the gametophyte, although there might be differences in conditions not yet tested.

We detected a striking expression of *PpSHI1* and *PpSHI2* in the axillary hairs of the gametophore, in the caulonemal filaments and in growing rhizoids during normal moss development. We also detected a GUS signal after wounding similar to that observed in the auxin signaling reporter line *GmGH3*$_{pro}$*:GUS* (Fujita et al., 2008).

GmGH3$_{pro}$*:GUS* has previously been shown to identify sites of high auxin signaling in moss (Bierfreund et al., 2003; Fujita et al., 2008). Staining is predominantly found in the apical and basal parts of the gametophore, the basal cells of the rhizoids and axillary hairs. A closer examination of the apical *GmGH3*$_{pro}$*:GUS* signal revealed a maxima in apically located axillary hairs. The overlap in expression between the *GmGH3*$_{pro}$*:GUS* reporter line and *PpSHI* suggests that *PpSHI1/2* are auxin responsive like *GmGH3*, or that *GmGH3* regulated expression depends on *PpSHI1/2* mediated induction of auxin signaling in *P. patens*. Because the *PpSHI2*$_{pro}$*:PpSHI2-GUS* line did not respond with any increase in GUS signal intensity after auxin treatment, we propose that *PpSHI2* is not an auxin responsive gene but that it is required for establishing high auxin signaling.

3.9.2 A putative role for axillary hairs in auxin production and distribution

The axillary hairs of mosses are small organs with unknown function. that are at least structurally similar to stipules of higher plants. *YUC*, *SHI/STY* genes and auxin signaling markers are all expressed in the stipules of *A. thaliana* (Chang et al., 2007; Aloni et al., 2003; Kuusk et al., 2002) suggesting that they are sites of auxin biosynthesis. We showed that both *SHI/STY* genes of *P. patens* are strongly expressed in the axillary hairs and we could also show that the number of axillary hairs increased in *PpSHI1/2* loss-of-function mutants, possibly to compensate for a reduced PpSHI function. Although no function for the axillary hairs in *P. patens* has been established it is known that axillary hairs in other moss species produce mucilage (Ligrone 1986). The mucilage is a slimy substance that has been proposed to protect the young apical part of the gametophore from dehydration (Schofield and Hebant, 1984). One bold hypothesis is that mucilage mediated diffusion would transport auxin since long range PAT could not be detected in the moss gametophore stem (Fujita et al., 2008). As mentioned in section 1.9.2, it has been shown that most of the auxin produced by protonemal filaments is found in the surrounding media, (Reutter et al., 1998), supporting a model where newly synthesized IAA is secreted and affects neighboring cells after diffusion rather than polar transport. It would be interesting to establish whether this putative IAA secretion also apply to the gametophore and specifically to the axillary hairs. It remain to be established how moss transport IAA and the characterization of the four *P. patens* homologues to the *PIN-FORMED* (*PIN*) gene family, as well as homologues to *A. thaliana* AUX1 auxin influx carriers, will be of great importance for future studies on auxin biology in moss.

3.9.3 *SHI/STY* mediated regulation of auxin biosynthesis appear conserved through evolution

In order to analyze the effects of constitutive *PpSHI1* expression on auxin signaling, we transformed the *GmGH3*$_{pro}$*:GUS* line (Bierfreund et al., 2003) with a *35S*$_{pro}$*:PpSHI1* construct. The construct was inserted into the BS213 locus by homologous recombination, resulting in several lines having severe developmental defects. These defects were positively correlated to *PpSHI1* expression and negatively correlated to *PpSHI2* expression, suggesting a negative feed-back loop, where *PpSHI1* negatively regulates *PpSHI2*.

In short, the *35S*$_{pro}$*:PpSHI1 GmGH3*$_{pro}$*:GUS* lines had reduced chloronema to caulonema ratio. The lines also showed signs of necrosis or premature ageing, an effect seen exclusively in older leaves and filaments but not in apical chloronemal filaments and in newly formed leaves.

Three out of the four analyzed lines showed a significant increase in IAA levels accompanied by a slight increase in levels of the terminally inactivated IAA catabolite oxIAA. This result strengthens our hypothesis that the ancestral function for SHI/STY members was transcriptional regulation of auxin homeostasis. Finally, these lines also showed a gradually increased GUS staining intensity, and expression of GUS in areas not reported as auxin signaling zones (Bierfreund et al., 2003). We thus propose that ectopic expression of *PpSHI1* in the gametophyte results in increased auxin signaling in tissues normally displaying auxin signaling, as well as auxin signaling in tissues normally not showing signs of auxin signaling. The developmental defects in older leaves and filaments could be explained by an increase of IAA levels and because IAA normally is not synthesized in these tissues, this would have dramatic effects. The same argument could be used to explain the apparently normal young leaves of the *35S*$_{pro}$*:PpSHI1 GmGH3*$_{pro}$*:GUS* lines as the young leaves potentially are subjected to higher endogenous IAA levels, as most data points towards an IAA production in the nearby axillary hairs.

3.9.4 *PpSHI*-function in caulonema, stem and rhizoid development

Two *PpSHI1* and two *PpSHI2* loss-of-function mutants, named *Ppshi1-1*, *Ppshi1-2*, *Ppshi2-1* and *Ppshi2-2*, were created by disrupting the native loci via homologous recombination. All mutants showed identical phenotypes such as reduced stem length and longer rhizoids. Increased relative amount of CK, in relation to auxin, could potentially lead to enhanced cell division and thus rhizoid length, and because exogenous auxin promotes stem elongation (Ashton et al., 1979) the reduced stem length may result from reduced IAA levels. The number of leaves was not affected suggesting that it

is only a reduction of stem internode elongation that causes the dwarf phenotype of the gametophores. We were also able to show that the mutants respond to exogenous auxin, by restoring stem length to that of wild type, verifying intact perception and signaling mechanisms.

Colonies of the mutant lines were compact and displayed a reduced area. They also had a reduced number of caulonemal filaments, compared to wild type. This could be due to suppressed caulonema formation and because exogenous auxin stimulates caulonema formation (Ashton et al., 1979) this phenotypic effect may also result from reduced auxin levels.

Taken together, our data indicate that the *SHI/STY* loss-of-function mutants in moss show developmental defects opposite of what we can see for auxin treated wild type moss. This support our hypothesis that the biological function of *SHI/STY* members in moss is to regulate development and growth by modulating auxin levels.

3.9.5 Auxin biosynthesis pathways in moss

Because SHI/STY proteins regulate auxin biosynthesis in *A. thaliana*, and likely also in *P. patens*, we wanted to study the genetic framework for auxin biosynthesis in moss.

To establish whether the Trp-dependent pathways for IAA biosynthesis in seed-plants are present also in mosses we searched the *P. patens* genome for *YUC*, *TAA1* and *CYP79B2/3* homologues. Rensing et al. (2008) reported six putative *YUC* homologues in moss. Accordingly, we found six *YUC* homologues clearly related to the *A. thaliana YUC* members. Only one of these genes has EST support, but our own expression analysis showed that at least five *YUC* homologues are expressed in *P. patens*. We also found four homologues to *TAA1/TAR1/TAR2* and two homologues to *TAR3/TAR4*. And finally, as previously reported by (Nelson, 2006), we could not find any loci in *P. patens* corresponding to putative *CYP79* members.

As mentioned in the introduction, previous studies have analyzed the effects of IAA precursor-feeding to moss filamentous tissue (Lehnert, 1982; Lehnert and Bopp, 1983). The authors suggested that the TAM pathway, mediated by YUC flavin monooxygenases in *A. thaliana*, was not present in moss filamentous tissue. Also IAN was completely ineffective as IAA precursor in the study by Lehnert and Bopp (1983). Although its function is unclear, we have found one NIT homologue in the *P. patens* genome. The NIT and YUC homologues in moss suggest that there might be alternative explanations to why TAM and IAN are ineffective in the bio-assays performed by Lehnert and Bopp (1983).

Even though previous reports (Lehnert and Bopp, 1983) have suggested differences between IAA biosynthesis pathways in bryophytes and angiosperms the genome sequences does not indicate any major differences. It is possible that some of the differences that do exist e.g. the lack of CYP79 members, could be explained by the lack of certain synthesis pathways partially integrated with IAA biosynthesis, e.g. the pathways for production of IGs or camalexin. As previously mentioned, glucosinolates are only found in *Brassicaceae* and related families (reviewed in Kliebenstein et al., 2005), and to our knowledge no CYP71B15/PAD3 homolog, that could indicate synthesis of camalexin, has been identified in moss. However, it should be noted that the moss genome contain several CYP71 members and one of these could have a function related to that of CYP71B15 or CYP71A13 in production of antimicrobial secondary metabolites.

We have not found the putative STY1 binding site, at a position similar to the STY1 binding sites found in *YUC4* or *ORA59* of *A. thaliana*, in any of the IAA biosynthetic gene in moss that we have analyzed. However, we have not yet tested experimentally whether PpSHI and STY1 proteins recognize identical promoter elements.

4 Conclusions

Characterization of higher order mutants of *SHI/STY* family genes suggest that the *SHI/STY* family act highly redundantly, but there are evidence of sub-functionalization.

SHI/STY family members have established functions in regulation of development of cotyledons, floral organs and leaves of higher plant.

SHI/STY proteins are DNA binding transcription factors activating transcription of targeted genes.

STY1 directly activate expression of *YUC4*, and thus activate auxin biosynthesis.

STY1 also appear to directly activate *YUC8* and *ORA59*, both having putative functions in auxin biosynthesis.

Transcription of *SHI/STY* genes is regulated via a GCC-box-like regulatory element, and the transcription factor DORNRÖSCHEN-LIKE, is a potential upstream regulator together with other members of the AP2/ERF family.

A *SHI/STY* function in activation of IAA synthesis appears conserved and it is likely that a *SHI/STY* member regulated IAA mediated developmental processes in the ancestral embryophyte.

5 Future perspectives

STY1-mediated regulation of development in A. thaliana

I have mainly been interested in understanding how auxin biosynthesis can be regulated at the transcriptional level and how this regulation affect postembryonic development of plants. For this I have studied the SHI/STY family of transcriptional regulators of auxin biosynthesis. There are however several question marks regarding the biological role for *SHI/STY* family members. E.g. is regulation of auxin biosynthesis the primary role for the *SHI/STY* gene-family or are the developmental abnormalities in SHI/STY multiple mutants a result of something else than only impaired auxin biosynthesis? Expression of the prokaryotic IAA biosynthesis gene *IaaM* by the *STY1* promoter in e.g. the *sty1 sty2* double mutant or the quintuple mutant could show if reduced auxin levels is the main reason for the developmental abnormalities. Another way would be to identify additional direct downstream targets and analyze their connection to auxin biosynthesis. We have already started to further characterize the *SHI/STY* family members by analyzing down stream targets in more detail. It would also be interesting to do ChIP-sequencing to find additional direct downstream targets.

Can *SHI/STY* or *ORA59* knock-out/down mutants suppress the *sur1* and *sur2* high auxin phenotypes like the *wei2* and *wei7* Trp-synthesis mutants? If yes, it would suggest that the IAOx-pool created by SHI/STY members is not only mediated by the YUC pathway, but must also at least partially be created by CYP79B2/3, It also suggest that SHI/STY members regulate Trp inflow to both these pathways. It could also verify that *ORA59*-derived Trp is used for IAA biosynthesis, in addition to formation of secondary metabolites.

Another unsolved issue is the biological function of *SHI/STY* gene expression in the lateral root primordia. This might however be resolved by simply adding additional *SHI/STY* mutations in the already existing quadruple mutant.

It would also be interesting to know if there are direct connections between *SHI/STY* members and other genes with similar functions, putatively being regulated by, or regulating, *SHI/STY* members, i.e. *LUG*, *DRN/DRNL* and *TOP/NGA,* and if stimuli e.g. light quality regulating other auxin biosynthesis pathways also can regulate *SHI/STY* members.

SHI/STY function in basal embryophytes

In addition to characterize the *A. thaliana SHI/STY* genes in more detail it would be interesting to study the ancestral role of auxin biosynthesis by further establishing a biological function for *SHI/STY* genes in less complex plants, like moss or liverworts. This is especially interesting considering the possibility of different auxin transport and homeostasis systems in bryophytes compared to higher plants. It would also be interesting to analyze the genes encoding for auxin biosynthetic enzymes in these organisms because very little related to auxin biosynthesis in bryophytes is known.

It would also be of interest to characterize *SHI/STY* genes and auxin mediated developmental processes in *Marchantia*, a group of liverworts thought to be the closest living relatives to the ancestral embryophyte.

Regulation of auxin biosynthesis

To fully understand the regulation of auxin biosynthesis and the cross-talk between different biosynthetic pathways we need to know the sub-cellular localization of the different pathways. It would be interesting to know if CYP79B2/3 is in the chloroplast and/or in another compartment e.g. the ER membrane. Because YUC6 has been shown to be cytoplasmic, but putatively associated with a small membrane structure (Kim et al., 2007), it would be interesting to establish a more detailed view of the localization of YUC members. Interaction studies using YUC4 could reveal proteins acting as post-translational modifiers/regulators, thus directing YUC4 localization. Interaction studies could also identify enzymes operating in a catalytic complex with YUC members and thus reveal the so far uncharacterized enzymes catalyzing the formation of TAM and/or IAOx in the YUC pathway.

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